Supplemental information

Characterization of Induced Neural Progenitors from Skin Fibroblasts by a Novel Combination of Defined Factors

Changhai Tian[1,2,5*], Qiang Liu[6], Kangmu Ma[2,5], Yongxiang Wang[2,5], Qiang Chen[2,5], Randall Ambroz[2,5], David L. Klinkebiel[4,5], Yuju Li[2,5], Yunlong Huang[2,5], Jianqing Ding[7], Jie Wu[6], Jialin C. Zheng[2,3,5*]

Supplemental Method

DNA methylation analysis

Bisulfite treatment was carried out using 1000 ng of sample genomic DNA and the EZ DNA Methylation-Direct kit (Zymo Research, Orange, CA). This process deaminates unmethylated cytosine residues to uracil leaving methylated cytosine residues unchanged. To perform PCR reactions, 32 ng of bisulfite-modified DNA was used as a template. The PCR reactions were performed in a total volume of 25 µl for 35 cycles using Roche Diagnostic Corporation (Indianapolis, IN) FastStart Taq DNA Polymerase (1.0 U), $MgCl_2$ solution (3.5 mM), dNTP's (0.2 mM), sense primer (0.24 µM), antisense primer (0.18 µM) (**Table S2**), with denaturation at 95 ℃ for 30 seconds, annealing temperature for 45 seconds as indicated in Table 1, and extension at 72 ℃ for 1 minute. Initial bisulfite sequencing primer sets, Nested 1 and 2 (**Table S2**) were used to bridge the Rat Nestin and Human herpes virus 1 thymidine kinase sequence of the construct to allow for proper discrimination of construct from endogenous mouse Nestin gene. This PCR product was used as the template for the internal pyrosequencing primer sets SEQ1, SEQ2 and SEQ3 (**Table S2**). All PCR products were electrophoresed on 0.8% agarose gel, stained with ethidium bromide, and visualized for appropriate and pure product before proceeding with all analyses using a Bio-Rad Laboratories (Hercules, CA) Gel-Doc UV Illuminator. Methylation percentage of each CpG was determined using a Qiagen (Valencia, CA) Pyromark Q24 pyrosequencer and sequencing primer indicated in Table S2, according to manufacturer recommendations.

Table S1: Primer pairs used for Real-time RT-PCR analyses of Transgenic and Endogenous genes

Target	Forward Primer	Reverse Primer	Annealing Temp.
Brn2 (tg)	AGCTGGAGAAGGAGGTGGTGAGAG	TTTATCGTCGACCACTGTGCTGG	60 ℃
Sox2 (tg)	CCTCCGGGACATGATCAGCATGTA	TTTATCGTCGACCACTGTGCTGG	60 ℃
Bmi1 (tg)	ATGAATGGAACCAGCAACAGCC	TTTATCGTCGACCACTGTGCTGG	60 ℃
Nr2e1 (tg)	CTCCTGTTGCTTTTACCAGCTTTACG	TTTATCGTCGACCACTGTGCTGG	60 ℃
c-Myc (tg)	TTTGCCCTGCGTGACCAGATC	TTTATCGTCGACCACTGTGCTGG	60 ℃
Brn2 (endo)	AGCTGGAGAAGGAGGTGGTGAGAG	CACCTGCTACCTGATATAGGATAGTCCAGTG	60 ℃
Sox2 (endo)	CCTCCGGGACATGATCAGCATGTA	CGGCATCACGGTTTTTGCGT	60 ℃
Bmi1 (endo)	ATGAATGGAACCAGCAACAGCC	GGAAGCAAACTGGACGACAGTCAC	60 ℃
Nr2e1 (endo)	CTCCTGTTGCTTTTACCAGCTTTACG	GGATCTTCCAGGTCAGAGGCAGAA	60 ℃
c-Myc (endo)	TTTGCCCTGCGTGACCAGATC	CCCAGCCAAGGTTGTGAGGTTAG	60 ℃

Table S2: Pyrosequencing primer sequences.

Region	Sense Primer	Anti-sense Primer	Sequencing Primer	PCR (bp)	Anneal (°C)
Nested 1	TTAGATGGTAGTGTGGATAAAAGGTAA	ATCCCCATAAAACCTAAAACCCAA		425	60
SEQ 1	TGTGGATAAAAGGTAATAATTAGTATG	CACTTATATCAAACTCCTCAAATCAATC	TAAAAGGTAATAATTAGTATGA	116	60
Nested 2	AGGTTATYGGTTTTGGTTTTGGGTGGG	TCACCTTAATATACRAAATAAACCT		735	64
SEQ 2	AGAGATTGATTTTGTAGTGGTAGGATA	ATCCCCATAAAACCTAAAACCCAA	TTATTTTAGATTAGTTTGGTTGG	163	60
SEQ 3	GAYGGGGTATAGGTATATTATTTTGTTA	AACTAAAAACATACAAATATATTTCTTC	TATAGGTATATTATTTTGTTA	149	56

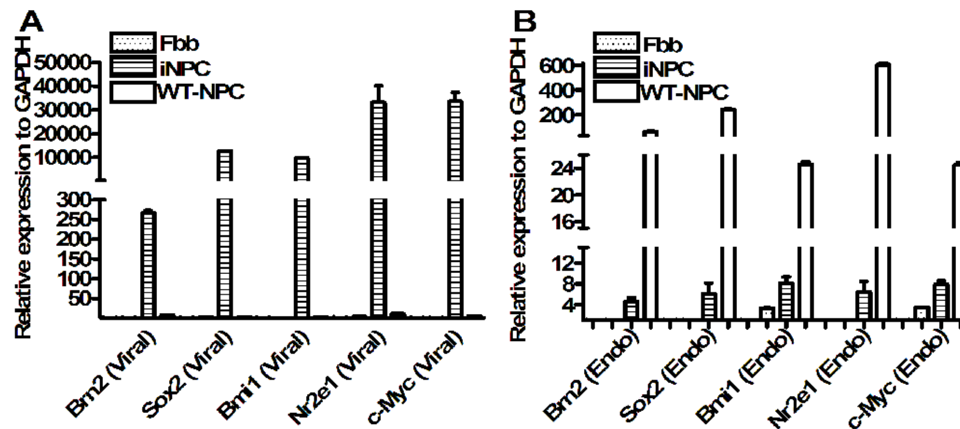

Fig. S1: SYBR-Green-based quantitative Real-Time PCR analyses of transgenic and endogenous genes in iNPCs. (**A**) Real-time RT-PCR analysis of iNPC line (P5) employing specific primer pairs for the detection of transgenic genes used for iNPC generation including Brn2, Sox2, Bmi1, Nr2e1 and c-Myc. (**B**) Real-time RT-PCR analysis of iNPC line (P5) employing specific primer pairs for the detection of endogenous genes. GAPDH-specific primer pairs were used as internal control; Fbb were used as negative control and WT-NPCs were used as positive control for endogenous detection.

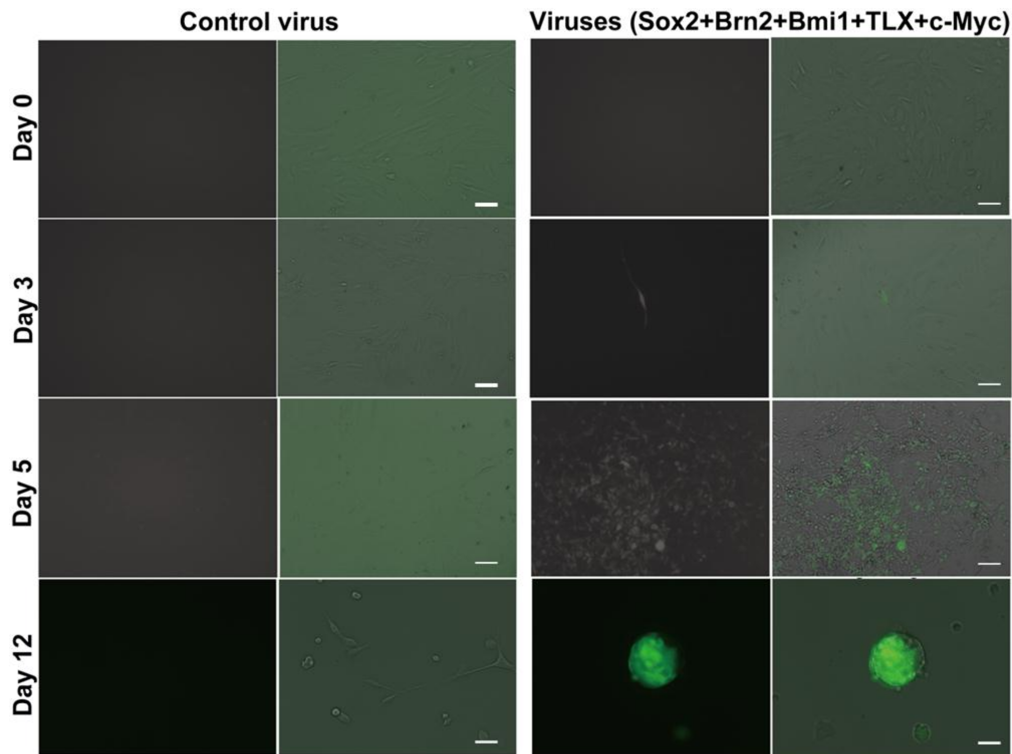

Fig. S2: Kinetics of direct conversion of skin fibroblasts into neural progenitor cells. Skin fibroblasts from adult E/Nestin:EGFP transgenic mice (provided by Richard J Miller from Northwest University) aged 5.5-7.0 weeks were retrovirally transduced with Brn2, Sox2, Bmi1, Nr2e1 (TLX) and c-Myc twice, and then cultured with NeuroCult® NSC Basal (Stem Cell Technologies, Inc., Canada) Medium supplemented with NeuroCult® NSC Proliferation Supplements (Stem Cell Technologies, Inc.), 20 ng/ml basic fibroblast growth factor (bFGF, BioWalkersville), 20 ng/ml Epidermal growth factor (EGF, BioWalkersville). Cells were subjected to regular fluorescence microscope at day 0, 3, 5 and 12. (Scale bars: 50 μm)

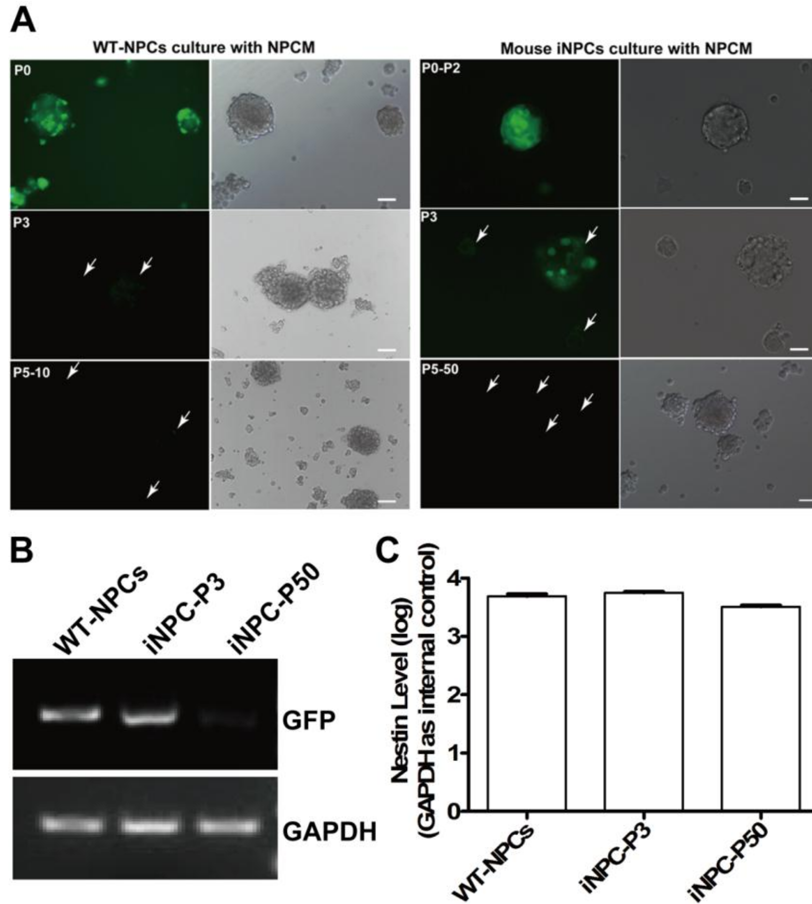

Fig. S3: EGFP expression in early and late passage of iNPCs. (**A**) iNPCs from skin fibroblasts and WT-NPCs from the brain of E/Nestin:EGFP transgenic mice (E14.5) were cultured with NPCM (NeuroCult® NSC Basal Medium supplemented with NeuroCult® NSC Proliferation Supplements, 20 ng/ml bFGF, 20 ng/ml EGF), and then subjected to fluorescence microscope at different passages. (Scale bars: 50 μm). (**B**) mRNA was extracted from early (P3) and late (P50) passage of iNPCs, respectively, and then subjected to RT-PCR with EGFP primer pair (5' primer: GCACGACTTCTTCAAGTCCGCCATGCC;3'primer:GCGGATCTTGAAGTTCACCT TGATGCC). (**C**) mRNA levels of endogenous nestin in WT-NPCs and iNPCs were analyzed by SYBR-Green based quantitative RT-PCR with specific nestin primer pair (see **Table 1**).

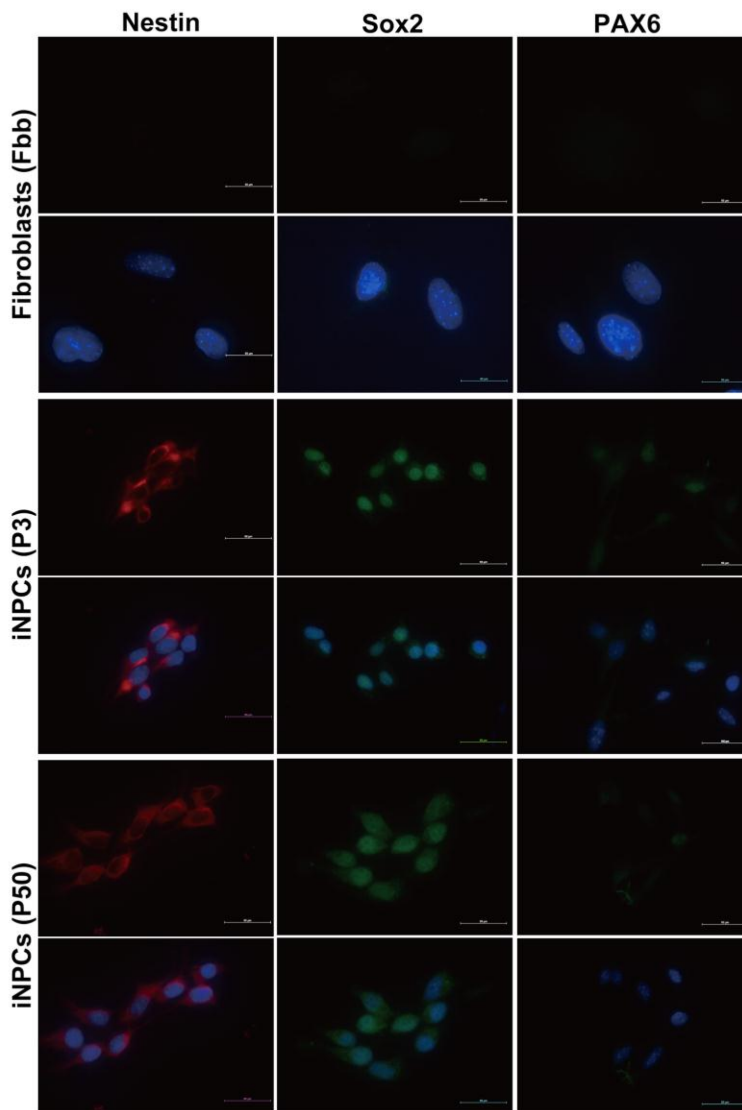

Fig. S4: Immunostaining analyses of NPC markers in Fbb, iNPCs. Fbb and iNPCs (P3 and P50) were plated on Poly-D-Lysine/Fibronectin-coated coverslips and cultured with NPCM and DMEM (supplemented with 10% FBS, 1% non-essential amino acid and 1% penicillin/streptomycin), respectively, and then fixed with 4% paraformaldehyde and subjected to immunostaining with Nestin (red), Sox2 (green), and Pax6 (green) antibodies, and nuclear staining with DAPI (blue). (Scale bars: 50 μm)

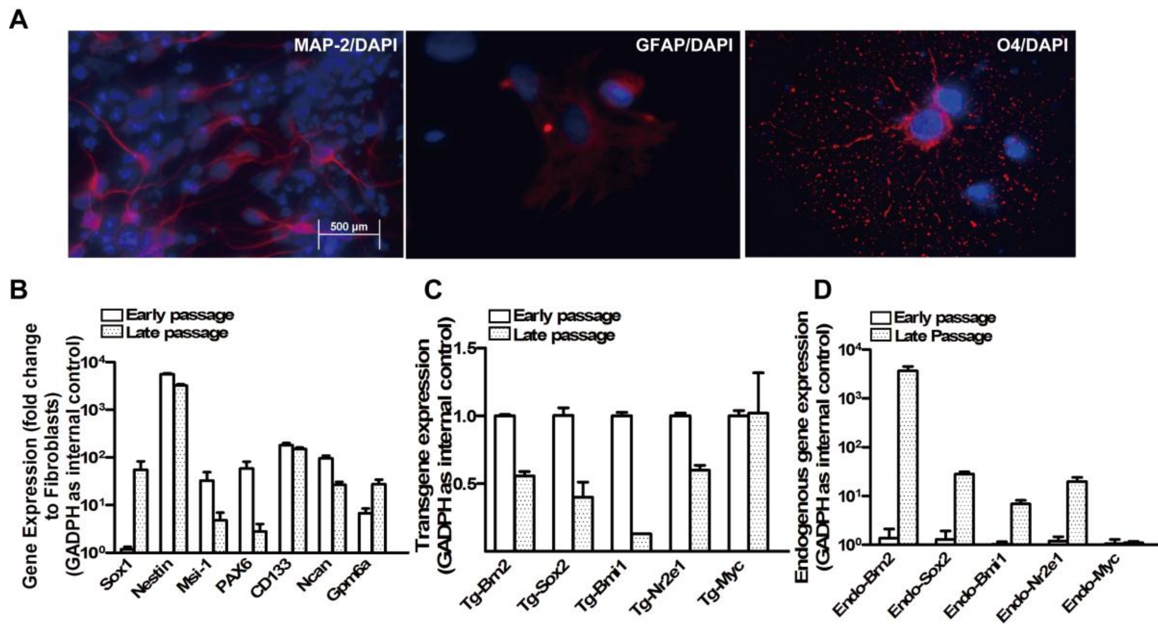

Fig. S5: Differentiation and gene expression in late passage of iNPCs. iNPCs in late passage were plated on Poly-D-Lysine/Fibronectin-coated coverslips and cultured with lineage differentiation media, respectively, and then fixed with 4% paraformaldehyde and subjected to immunostaining with MAP-2, GFAP, and O4 antibodies, and nuclear staining with DAPI (blue). (Scale bars: 500 μm) (**A**). mRNA was extracted from early and late passage of iNPCs, respectively, and then subjected to SYBR-Green-based quantitative Real-Time PCR analyses with specific transgene primer pair (**B**) and endogenous primer pair (**C, D**) (see **Table 1** and **Table S1**).

A

Nestin 2nd intron enhancer | Human TK promoter | EGFP | pA

5' — 3'

B

Linker

CG = SEQ1 CpG's

CG = SEQ2 CpG's

CG = SEQ3 CpG's

CGTTAAGACCTCTGCCCTGAAGAGTTTGTGATCCTGAGATGAGGGCTTTAGCCCCAGTCAGTCCTCTGAGGGGAAGG
GTCCAGGCAGCTCTGAGGAATGTAACCACTGGCGTTTGAGGTCTGAAAAGGATTTGGAGAAGGGGAGCTGAATTCAT
TTGCTTTTGTCTGTTACCAGCTCTGGGGGCAGAGAGAGAGCCATCCCCTGGGAACAGCCTGAGAATTCCCACTTCCC
CTGAGGAGCCCTCCCTTCTTAGGCCCTCCAGATGGTAGTGTGGACAAAAGGCAATAATTAGCATGAGAATCGGCCTC
CCTCCCAGAGGATGAGGTCATCGGCCTTGGCCTTGGGTGGGGAGGCGGAGACTGATCTGAGGAGTCTGATATAAGTG
TTAGCAATTCATTTGGCCCTGCCTCCGACTGTGGGAATCTGCATGTGGGGTCTCCCTGTGTCTCAAATATGGGTTGG
CTAAGTATATATCTGTGGGTATATGACTGTGTGGCTTTTATATGACAATGGTCACAATAGAGATTGATCCTGCAGTG
GCAGGACATGCTACCTCAGATCAGCTTGGCTGGCTCGGGCGGGTCGCGCGCCCCAGAGTGTGACCTTTTCGGTC
TGCTCGCAGACCCCCGGGCGGCGCCGCCGCGGCGGCGACGGGCTCGCTGGGTCCTAGGCTCCATGGGGACCG
TATACGTGGACAGGCTCTGGAGCATCCGCACGACTGCGGTGATATTACCGGAGACCTTCTGCGGGACGAGCCG
GGTCACGCGGCTGACGCGGAGCGTCCGTTGGGCGACAAACACCAGGACGGGGCACAGGTACACTATCTTGTCA
CCCGGAGGCGCGAGGGACTGCAGGAGCTTCAGGGAGTGGCGCAGCTGCTTCATCCCCGTGGCCCGTTGCTCGC
GTTTGCTGGCGGTGTCCCCGGAAGAAATATATTTGCATGTCTTTAGTTCTATGATGACACAAACCCCGCCCAGCGT
CTTGTCATTGGCGAATTCGAACACGCAGATGCAGTCGGGGCGGCGCGGTCCGAGGTCCACTTCGCATATTAAG
GTGACGCGTGTGGCCTCGAACACCGAGCGACCCTGCAGCGACCCGCTTAACAGCGTCAACAGCGTGCCGCAGA
TC

C

SEQ1	% CpG Methylation		
CpG Base Position	1	29	53
iNPC with GFP	7	11	24
iNPC without GFP	33	48	54

SEQ3	% CpG Methylation									
CpG Base Position	1	7	9	38	55	62	69	71	81	90
iNPC with GFP	10	13	14	23	16	18	16	8	17	20
iNPC without GFP	74	55	54	59	59	59	68	37	54	87

SEQ2	% CpG Methylation																
CpG Base Position	1	5	10	12	14	35	44	54	58	61	64	67	69	72	75	78	84
iNPC with GFP	19	20	12	25	16	29	13	10	13	7	9	7	5	10	6	14	16
iNPC without GFP	68	65	70	69	71	90	75	58	43	33	52	65	42	48	42	55	68

Fig. S6: DNA methylation analysis of transgenic promoter driving EGFP expression. (**A**) The schematic construct used for E/Nestin:EGFP transgenic mice. (**B**) The promoter sequence including rat nestin second intron enhancer, linker and human thymidine kinase (TK) minimal promoter, which was used to drive EGFP expression. Numbers indicate potential methylation sites from sequence 1 (SEQ1, blue color), sequence 2 (SEQ2, black

color) and sequence 3 (SEQ3, green color). (**C**) Genome DNA samples were extracted from iNPCs with EGFP and iNPCs without GFP, and then subjected to bisulfite treatment using EZ DNA Methylation-Direct kit. Methylation percentage of each CpG was determined using a Qiagen (Valencia, CA) Pyromark Q24 pyrosequencer and sequencing primer indicated in **Table S2**, according to manufacturer recommendations. Green and red colors represent lower and higher CG methylation levels, respectively.