Supplementary Material

Derivation of haploid neurons from mouse androgenetic haploid embryonic stem cells

He Xu, Chunmei Yue, Ting Zhang, Yuanyuan Li, Ao Guo, Jiaoyang Liao, Gang Pei, Jinsong Li, Naihe Jing

Materials and Methods

Mouse diploid and haploid ESC culturing

The mouse androgenetic haploid ESC line F9 was a gift from Dr. Jinsong Li'lab [1]. The mouse haploid Fucci-ESCs were established by inserting pFucci-G1 Orange and pFucci-S/G2/M Green plasmid into mouse androgenetic haploid ESC line HG165 (from Dr. Jinsong Li'lab) in Dr. Gang Pei's lab. The diploid mouse ESCs used were E14 and diploidized F9. Both mouse haploid and diploid ESC lines were cultured on a feeder layer in standard mouse ESC medium supplemented with 1000 U/mL LIF (Millipore), 1 μmol/L PD0325901 (Selleckchem) and 3 μmol/L CHIR99021 (Selleckchem). The medium was changed daily, and haESC was disaggregated and passaged by 0.05% Trypsin-EDTA every 3 to 5 days. Cells were maintained in an incubator at 37 °C with 5% CO_2.

FACS-enrichment of mouse androgenetic haploid ESC

The mouse androgenetic haploid ESCs were dissociated into single cells by Trypsin-EDTA, and then incubated with 15 μg/mL Hoechst 33342 in ESC medium in a 37 °C water bath for 15 min. Then the cells were spun down, rinsed with PBS once,

and re-suspended in ESC medium with 10 μmol/L ROCK inhibitor Y-27632. The cell suspension was then filtered through a 40 μm cell strainer. Haploid cells were sorted using the 355 nm laser in BD FACS Aria II (BD Biosciences). Normal diploid ESC sample was prepared as a negative control for each sorting. The collected haploid cells carrying 1-copy DNA were suspended with fresh neural differentiation medium containing 10 μmol/L ROCK inhibitor Y-27632, and started neural differentiation.

Neural differentiation of mouse androgenetic haploid ESC

The methods used to direct neural differentiation of mouse haploid ESC in a serum-free system is schematically summarized in Figure 1A. The mouse haESCs were induced as aggregates and suspended in 5% Knockout serum replacement (KSR) for 2 days, and then in N2B27 medium to form embryoid bodies (EBs) for 6 days. Finally EBs were dissociated into single cells at day 8 and replated in N2 medium for neuronal differentiation and maturation for another two weeks.

Single-cell real-time PCR

The day-8 EBs were dissociated into single cells by Accutase (Stemcell Technology) at 37 °C for 15 min. Then the single cells were subjected to FACS analysis, and sorted haploid cells were suspended and diluted in PBS-BSA (0.1% BSA in PBS) solution. The individual haploid cells were picked by micropipette and transferred into PCR tube containing 3.45 μL lysis solution, 1 μL of anchored oligo-dT primer (10 μmol/L), 1 μL of dNTP mix (2.5 m mol/L), 0.05 μL Rnase (40 U/μL) inhibitor and 1.4 μL of nuclease free water. RNA was denatured at 72 °C for 3 min and immediately placed on ice. Total volume of 6.55 μL first-strand reaction mix, containing SuperScript II

reverse transcriptase (100 U, Invitrogen), RNase inhibitor (10 U, Roche), Betaine (1 mol/L, Sigma-Aldrich), $MgCl_2$ (9 mmol/L, Sigma-Aldrich), LNA template switching oligos (TSO, 10 μmol/L) were added to each sample for reverse transcription. After the 1st-strand synthesis, cDNA was directly preamplified with KAPA HiFi Hotstart ReadyMix (KAPA Biosystems) by 5'-amine (NH2)-blocked IS-PCR primer for 19 cycles.

Immunofluorescence staining

Before immunofluorescence staining, sorted haploid neural stem cells were reseeded on glass coverslips for overnight or replated into PDL-coating 35 mm dishes for neuronal differentiation. For immunofluorescence staining, the cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized using 0.2% Triton X-100 in PBS for 15 min, and blocked in 5% BAS in PBS. Cells were incubated with the following primary antibodies anti-Oct4 (1:200, Santa Cruz), anti-Sox1 (1:200), anti-Pax6 (1:200, Millipore), anti-Tuj1 (1:500, Sigma), Anti-centromere (1:50, Antibodies Incorporated) overnight at 4 °C. As for secondary antibodies, the cells were treated with a fluorescently coupled secondary antibody and then incubated for 1 h at room temperature. DAPI was used for DNA staining, and all antibodies were diluted in blocking solution. The images were captured on Leica TCS SP5 and Leica TCS SP5 laser-scanning microscopy (Leica Microsystems). Images were collected at 1024×1024-pixel in resolution.

Modeling the diploidization dynamics of haploid cells

To model the diploidization kinetics of haploid cells during self-renew and neural

differentiation process, the percentage of haploid cells over total cells at each tested time-point were calculated. Due to the irreversible and spontaneous diploidization at different cell cycle stages, the haploid cells with 1-copy DNA set at G1 phase was sorted into 1c-peak, and the haploid cells with 2-copy DNA set at S G2/M phase, mixing with diploid cells at G1 phase, was sorted into 2c-peak (Figure 1).

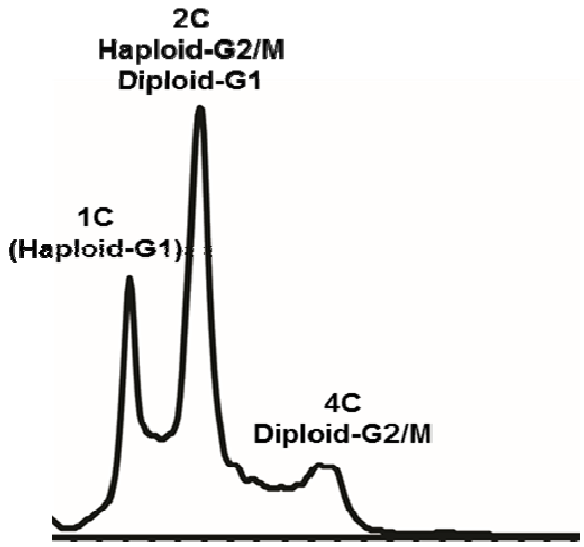

Figure 1. DNA content profiles of haploid cells

(1) If x% is the percentage of diploid cells at G1 phase in 2c-peak, the percentage of haploid S G2/M cells is:

$$\textit{Haploid cells in (S G2/M)} = 2C\% - x\%$$

The percentage of total haploid cells is thus:

$$\textit{Total haploid cells} = 1C\% + (2C\% - x\%)$$

(2) We assumed that the cell division cycles of diploid cells among haploid cells shown in Figure 1 is same as those of pure diploid cells shown Figure 2, then the proportion of diploid cells in 2c- vs 4c-peak should be similar between Figure 1

and Figure 2.

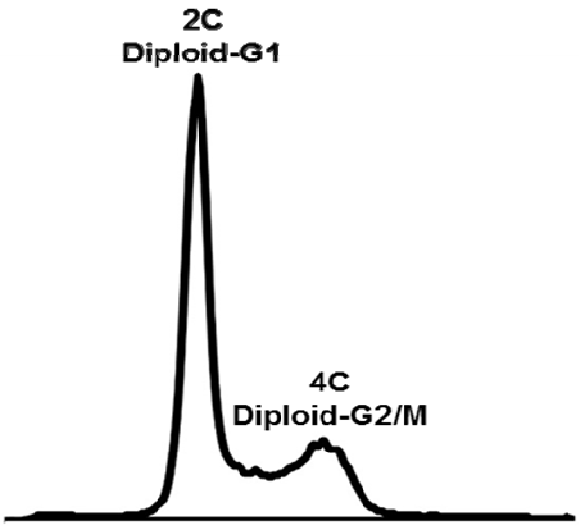

Figure 2. DNA content profiles of diploid cells

The ratio of x% to 4c% in Figure 1 is equal to the ratio of G1% to S G2/M% in Figure 2:

$$\frac{x\%}{4C\%} = \frac{G1\%}{S\ G2/M\%}$$

The percentage of diploid cells at G1 phase is thus:

$$x\% = \frac{G1\%}{S\ G2/M\%} * 4C\%$$

References

1. Yang H, Shi L, Wang BA, Liang D, Zhong C, Liu W, *et al.* Generation of genetically modified mice by oocyte injection of androgenetic haploid embryonic stem cells. Cell 2012, 149: 605–617.

S1

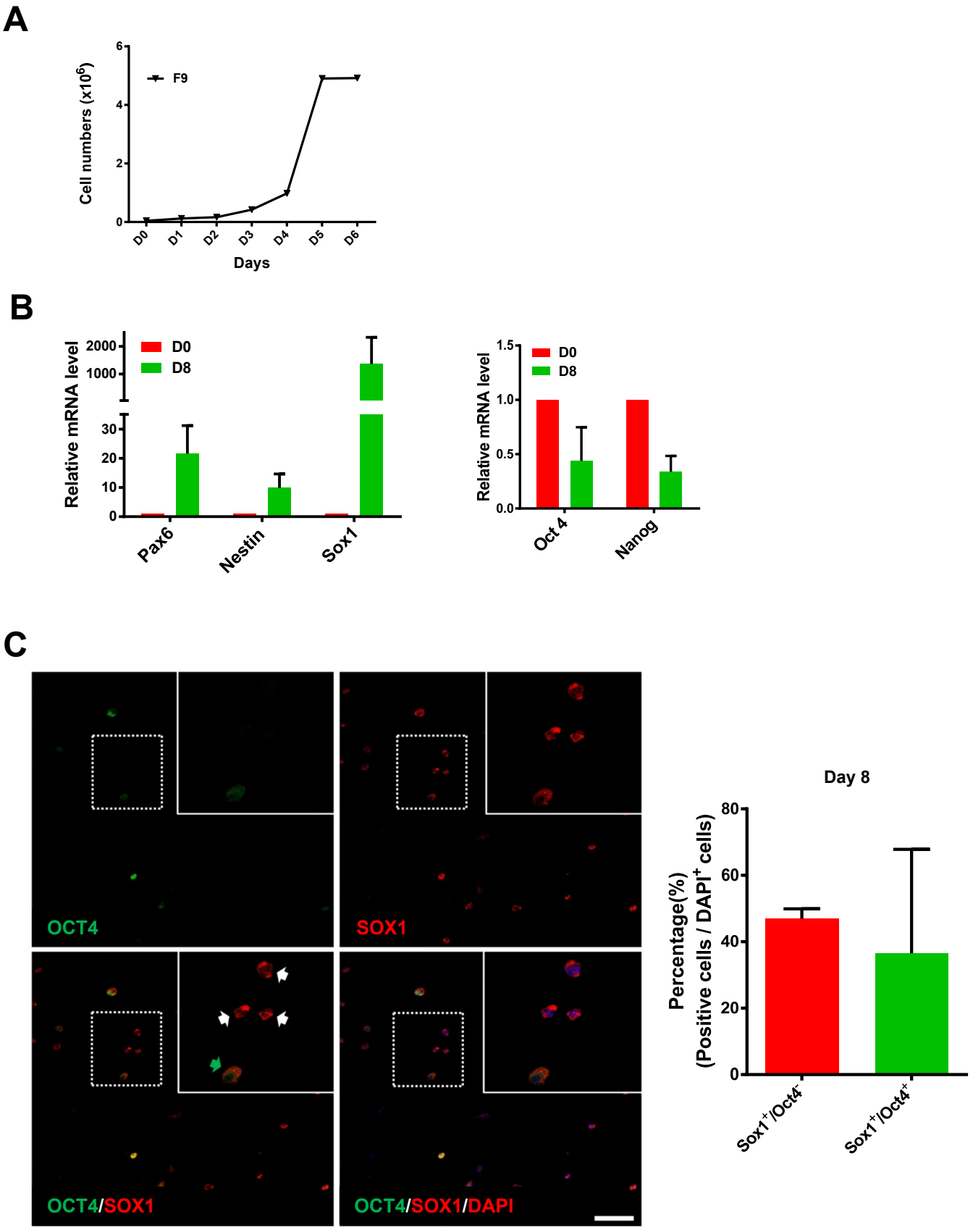
A
Cell numbers (x10^6)
F9
Days
D0
D1
D2
D3
D4
D5
D6
B
Relative mRNA level
D0
D8
Pax6
Nestin
Sox1
Oct 4
Nanog
C
OCT4
SOX1
OCT4/SOX1
OCT4/SOX1/DAPI
Day 8
Percentage(%)
(Positive cells / DAPI+ cells)
Sox1+/Oct4-
Sox1+/Oct4+

Figure S1 Derivation of haploid neurons from mouse androgenetic haploid embryonic stem cells (haESCs). (A) Growth curve of F9 haESCs. (B) mRNA expression of the neural stem cell genes *Pax6*, *Nestin*, and *Sox1* (left panel), and the pluripotent genes *Oct4* and *Nanog* (right panel) in haploid cells at days 0 and 4 during neural differentiation. (C) Co-staining of Oct4 and Sox1 in day-8 haploid cells (left panels; scale bars, 50 μm) and their quantification (right panel). The green arrow indicates SOX1-and OCT4-double positive cells, and the white arrows indicate the SOX1-positive cells. N = 3 independent experiments. All data are presented as the mean ± SD.

S2

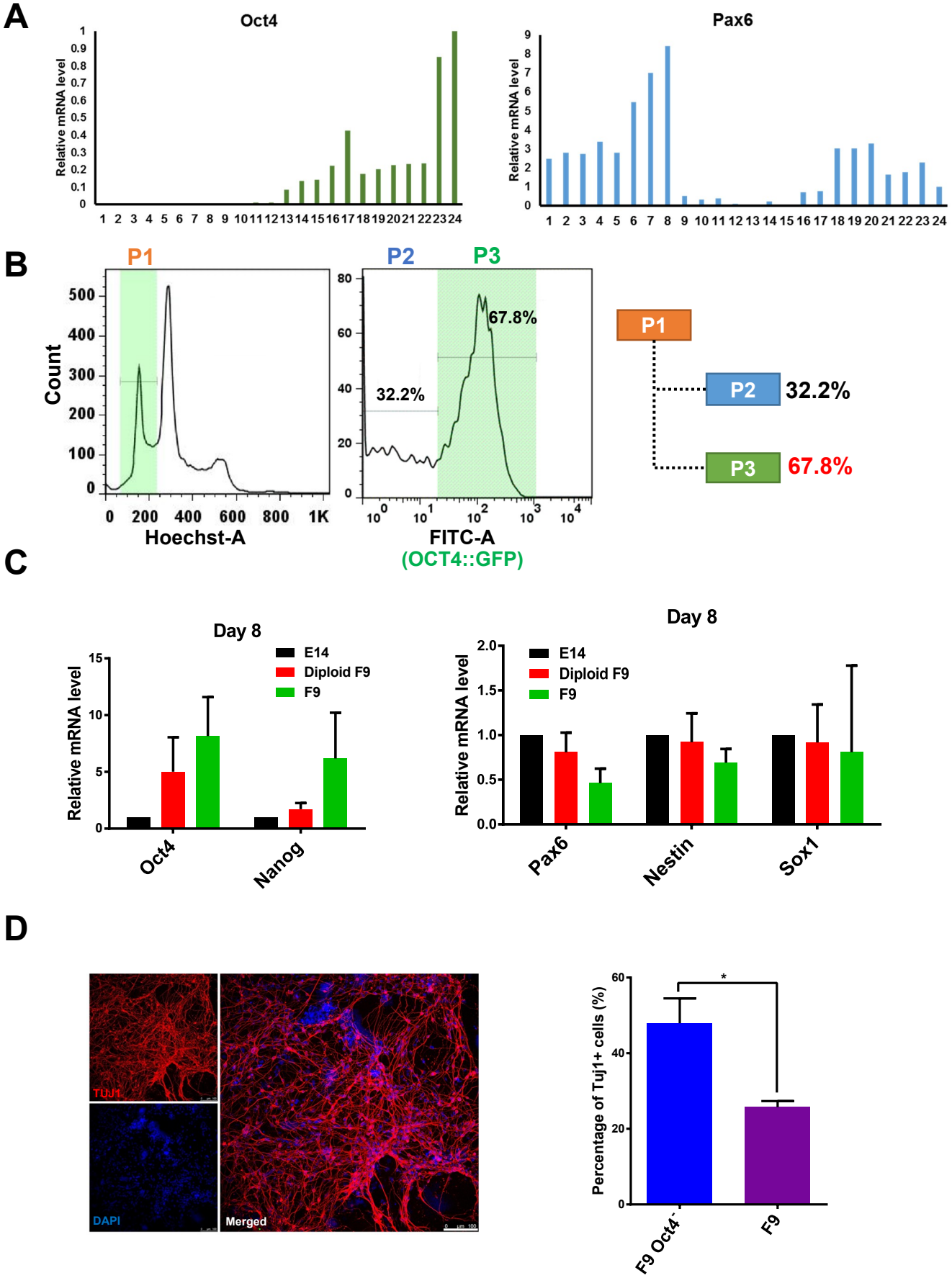
A
Oct4
Relative mRNA level
Pax6
Relative mRNA level
B
P1
P2
P3
Count
Hoechst-A
FITC-A
(OCT4::GFP)
67.8%
32.2%
P1
P2
32.2%
P3
67.8%
C
Day 8
E14
Diploid F9
F9
Relative mRNA level
Oct4
Nanog
Day 8
E14
Diploid F9
F9
Relative mRNA level
Pax6
Nestin
Sox1
D
TUJ1
DAPI
Merged
Percentage of Tuj1+ cells (%)
*
F9 Oct4-
F9

Figure S2 Derivation of haploid neurons from mouse androgenetic haploid embryonic stem cells (haESCs). (A) The mRNA expression of *Oct4* and *Pax6* in day-8 haploid cells by single cell-PCR. The sequential numbers on the horizontal axis indicate the numbering of each single cell. (B) Flow analysis of GFP-positive cells among day-8 haploid cells from among F9 cells. The cells in peak1 (P1) contain 1 copy of chromosomal set separated by DNA content, cells in P2 are the subgroup of P1 without GFP expression, cells in P3 are the subgroup of P1 with GFP expression. (C) mRNA expression of *Oct4*, *Nanog*, *Pax6*, *Nestin*, and *Sox1* in neural stem cells derived from diploid E14 ESCs, F9 diploid cells, and F9 haESCs. (D) Representative image of Tuj1 expression in day-12 neurons derived from $Oct4^-$ F9 cells (left panel; scale bar, 100 μm), and percentage of $Tuj1^+$ neurons among day-12 cells from $Oct4^-$ F9 and F9 cells (right panel; N = 3 independent experiments; all data are presented as the mean ± SD, two-tailed t test).

S3

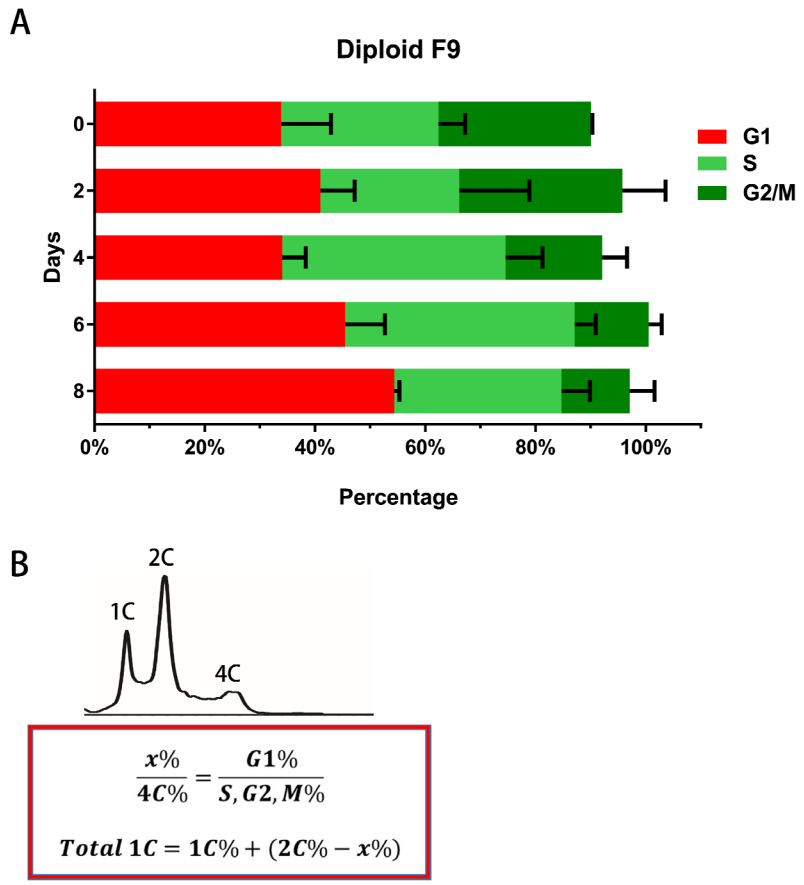

Figure S3 Derivation of haploid neurons from mouse androgenetic haploid embryonic stem cells (haESCs). (A) Percentage of cells in G1, S, and G2/M on days 0, 2, 4, 6, and 8 during neural differentiation of diploid F9 cells. (B) Mathematical model for calculating the percentage of G2/M-phase haploid cells in differentiated and undifferentiated haESCs. 1C, 2C, and 4C indicate cells containing one chromosomal copy (1C), two copies (2C) and 4 copies (4C).