Supplementary Data

Supplementary Methods

Three-step protocol for spontaneous differentiation of mouse induced pluripotent stem (embryonic stem) cells

Mouse induced pluripotent stem cells (iPSCs) were cultured on the feeder layer of mouse embryonic fibroblasts to maintain their stemness and dissociated using trypsin to obtain a suspension of single cells, which was then transferred for subculture or differentiation culture. We established a three-step protocol to investigate the spontaneous differentiation of embryonic stem cells (ESCs) or iPSCs in vitro.

First, to remove unwanted feeder cells from iPSCs, cells were plated in a T75 tissue culture flask, and the feeder cells were allowed to attach for 1–2 h in the incubator. The iPSCs were then collected and centrifuged at 1,000 rpm/min for 5 min. Most feeder cells were removed after this procedure was performed twice.

Second, for formation of embryoid bodies (EBs), two methods exist: suspension culture and hanging drop culture. For suspension culture, 10 mL of ESCs in suspension with a density of 1×10^5/mL was seeded into a 100-mm bacterial-grade dish. Every 3–4 h, the dish was shaken to ensure that cells did not attach to the plastic surface of the dish for the first 12 h. The cells then naturally stuck to each other and formed aggregates without any shaking. This method is the most basic method and has been utilized to initiate the differentiation of ESCs into various differentiated cell types. However, EBs formed from such ESCs have low homogeneity in morphology and differentiation. Hanging drop culture is partially performed the same as suspension culture; a suspension of single cells with a density of 10^5 cells/mL is plated in 20- to 30-μL drops on the lid of Petri dishes in regular arrays. A standard 100-mm dish can accommodate ~30–40 drops. The lid of the dish is then inverted and placed over the bottom of the dish, which is filled with 5 mL of phosphate-buffered saline to prevent the drops from drying. The cells are incubated for 2 days, during which they aggregate into a single EB; the EBs are then harvested and subsequently transferred to bacterial-grade dishes for 2–3 days of culture.

After 4–5 days of culture, the EBs are transferred onto a gelatin-coated dish and allowed to attach; the cells then expand and spontaneously differentiate into progenitor cells.

All-trans retinoic acid induced neural differentiation of mouse iPS (ES) cells

This protocol aims to differentiate mouse iPS (ES) cells into βIII-tubulin-positive neurons in vitro following a modified 4−/4+ differentiation protocol developed by Gottlieb et al. [1].

Formation of EBs (4− stage). Aliquot of 1×10^5 cells/mL in 10 mL of EB-formation medium was placed in a sterile 10-cm bacterial Petri dish as mentioned earlier; the medium was replaced every day.

Induction of neuronal differentiation (4+ stage). The neural induction medium was prepared by adding all-trans retinoic acid to yield 5 μM solution in EB-formation medium. The EBs in 10 mL of neural induction medium were resuspended and the suspension was transferred to a fresh Petri dish. The dish of cells was incubated at 37°C and 5% CO_2 incubator for an additional 4 days; the medium was changed every day.

Expansion of neuronal cells from EBs. After 4−/4+ stage, 8-day EBs are transferred onto gelatin-coated dishes in EB-formation medium for adherent culture. Neuronal-like cells and their processes extend and migrate from the attached EBs for another 48 h.

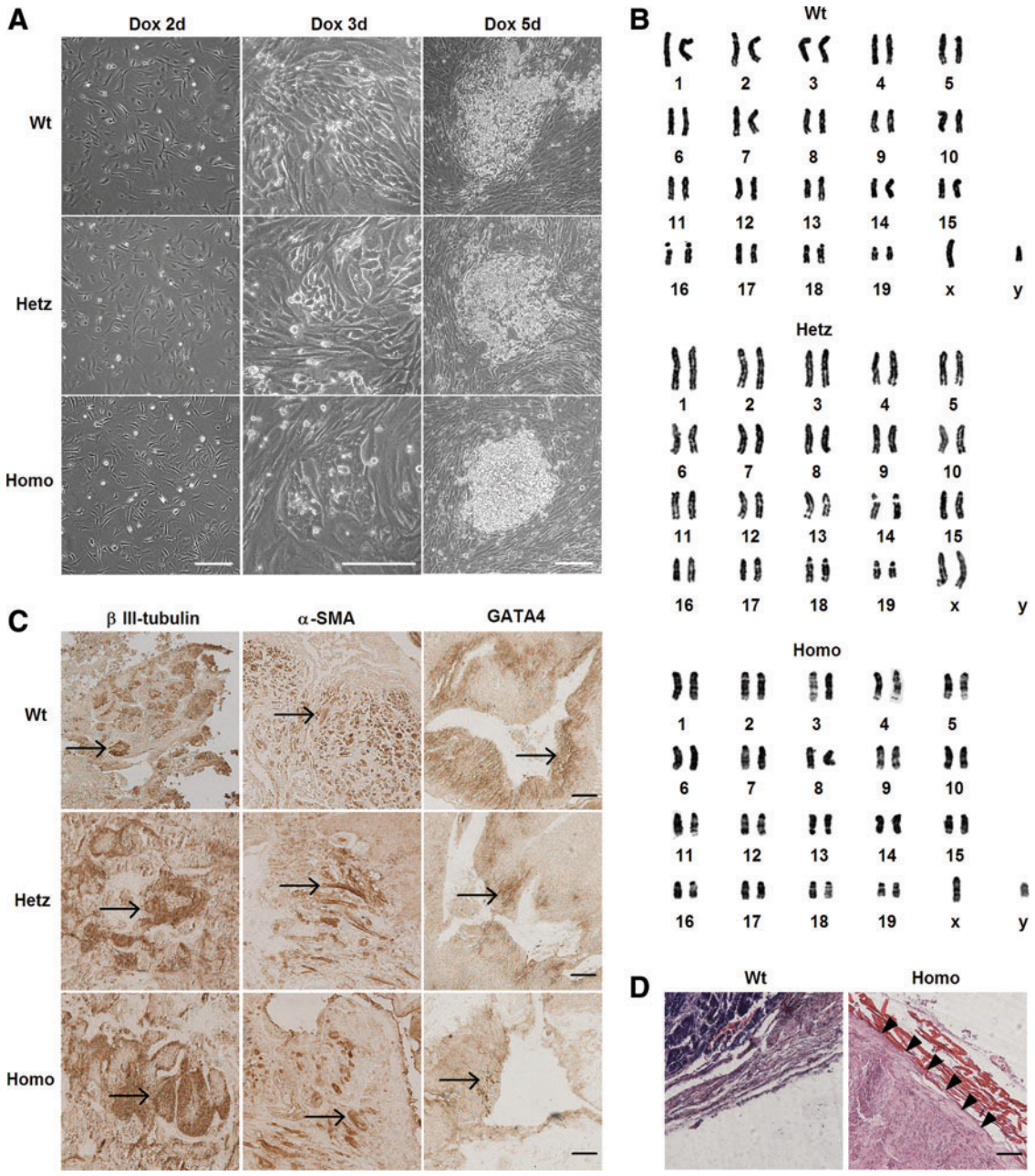
Immunohistochemistry

Immunohistochemistry of paraffin sections from teratomas was performed according to the manufacturer's recommendations (Bioss). Primary antibodies were diluted in blocking buffer and applied overnight at 4°C. Primary antibodies were used at the following dilutions: βIII-tubulin (1:500; Abcam), α-smooth muscle actin (1:200; Abcam), and GATA4 (1:200; Abcam).

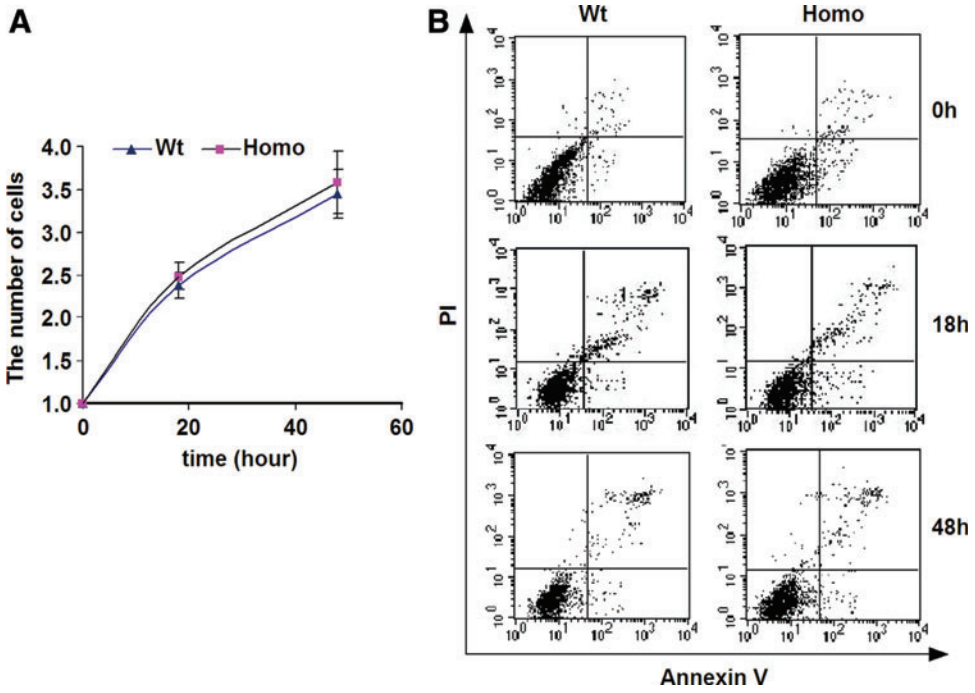
Characterization of iPSCs and EBs by reverse transcription–polymerase chain reaction and quantitative reverse transcription–polymerase chain reaction analyses

Total RNA was extracted using the RNeasy kit (Qiagen), and cDNA was generated using Superscript II (Invitrogen). Polymerase chain reaction was performed for 35 cycles for all marker genes.

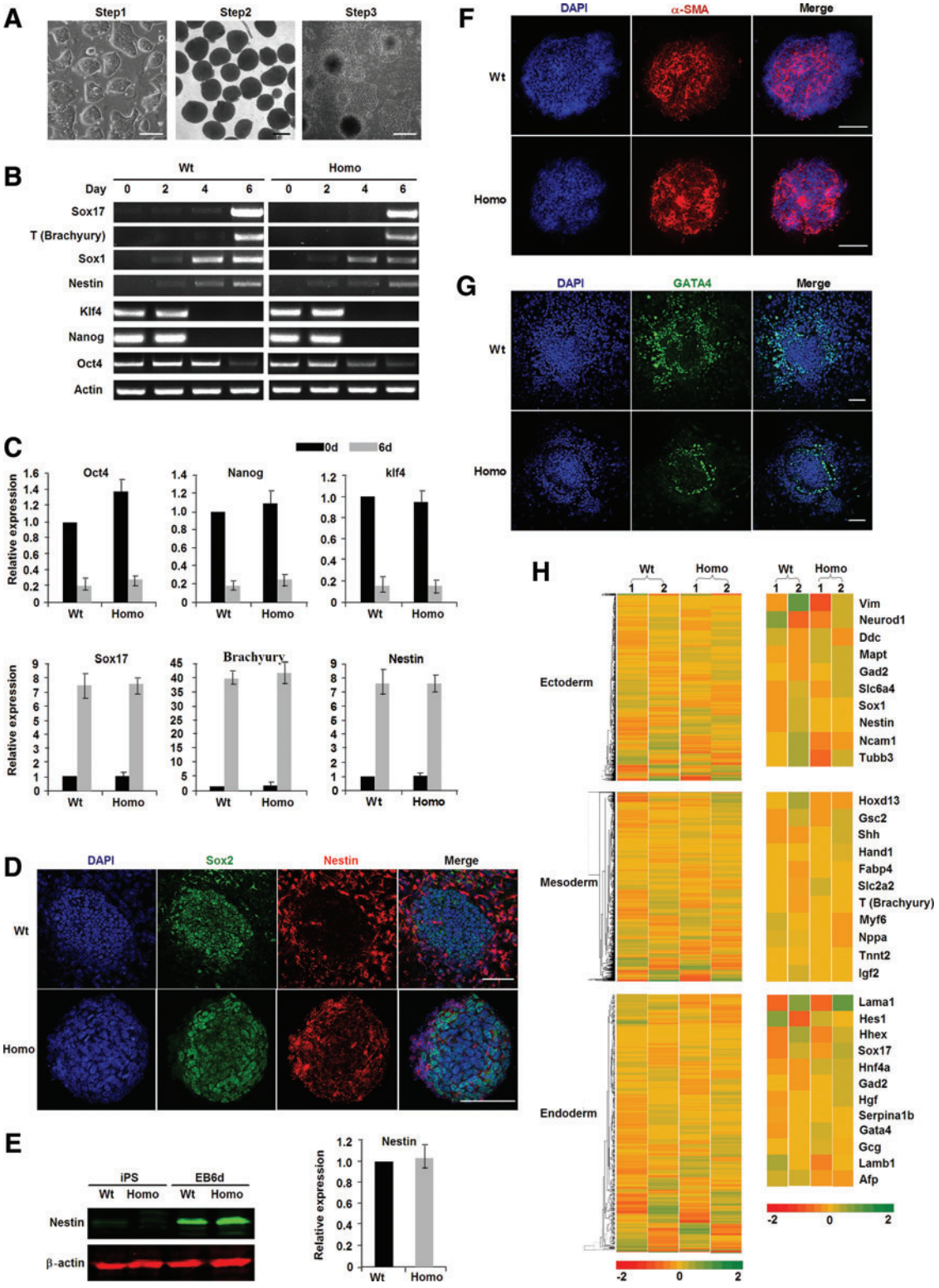
Oligonucleotides were designed for the detection of the following genes: *Oct4, Klf4, Nanog, Sox17, Foxa2, Brachyury, Nestin,* and *β-actin*. Quantification was normalized on the endogenous β-actin gene. The primers of these genes are displayed in Supplementary Table S1.



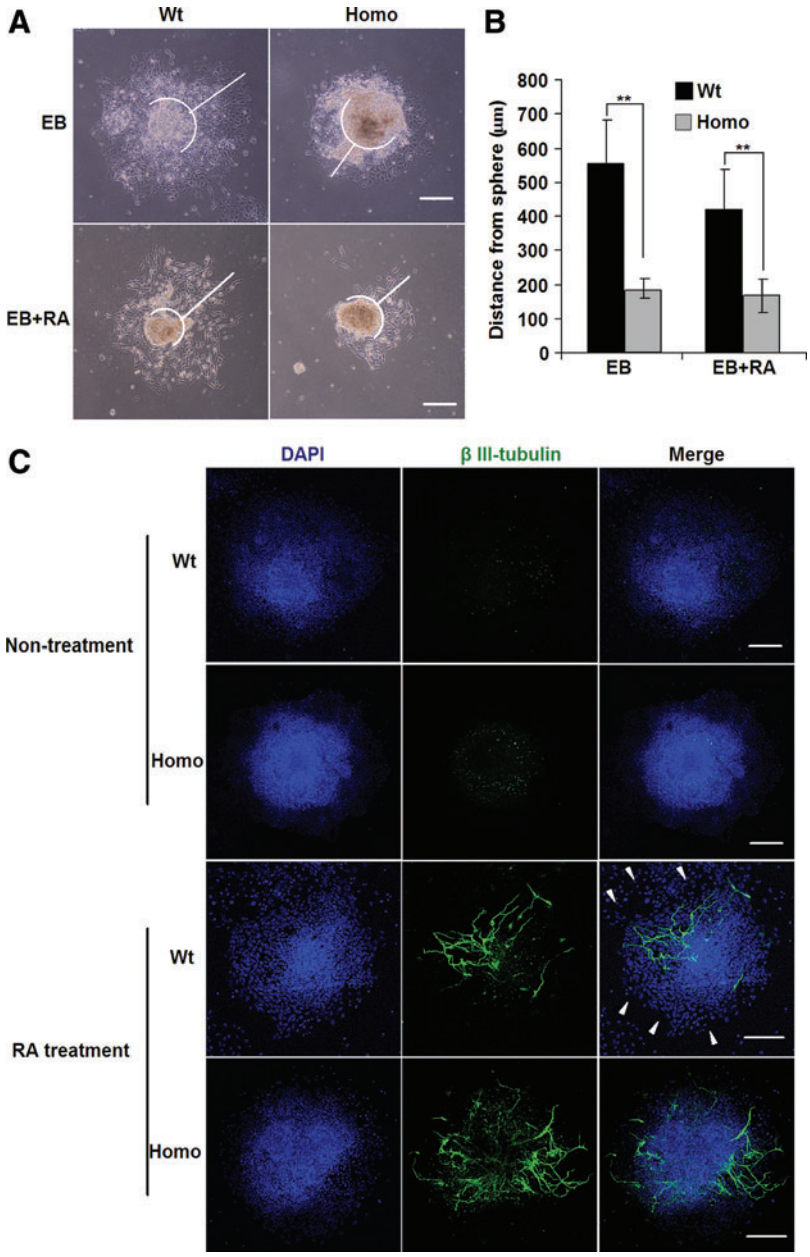
SUPPLEMENTARY FIG. S1. Characterization of induced pluripotent stem cells (iPSCs) derived from mouse embryonic fibroblasts (MEFs) of the three genotypes. **(A)** MEFs of the three genotypes from 13.5-day embryos were reprogrammed to iPSCs, and various morphologies were observed for cells at days 2, 3, and 5 after dox induction. Scale bar = 100 μm. **(B)** The chromosomal contents of iPSC lines were analyzed using high-resolution G-banding karyotypes. **(A)** Wt, 40, X + Y. **(B)** Hetz, 40, X + X. **(C)** Homo, 40, X + Y. A representative experiment is shown. **(C)** Three germ layer markers (βIII-tubulin, α-smooth muscle actin, and GATA4) displayed by immunohistochemistry in Wt, Hetz, and Homo teratomas. Scale bar = 100 μm. **(D)** Boundary of Wt and Homo teratomas determined by hematoxylin and eosin staining. The *arrows* in Homo teratomas section showed relatively intact borders. Scale bar = 100 μm.



SUPPLEMENTARY FIG. S2. Proliferation and apoptosis in wild-type (Wt) and homozygous embryoid bodies (Homo-EBs). **(A)** Cell counts at different time points in day-4 EBs (0 h, att-18 h, and att-48 h) were not significantly different between Wt and Homo. **(B)** Cell apoptosis tested by flow cytometry demonstrated no significant differences in propidium iodide and Annexin-V staining between the two groups. 0 h: day-4 EBs without attachment to the dish; att-18 h: day-4 EBs attaching to gelatin-coated dish for another 18 h; att-48 h: day-4 EBs attaching to gelatin-coated dish for another 48 h. A representative experiment is shown; the experiment was repeated three times.



SUPPLEMENTARY FIG. S3. In vitro EB formation and spontaneous differentiation. **(A)** Process of development from iPSCs to EBs: Step 1, iPS colonies without MEFs; Step 2, EB suspension culture; Step 3, EBs attached to gelatin dishes at 4–5 days. Scale bar = 500 μm. **(B)** Reverse transcription–polymerase chain reaction analysis of embryoid stem markers (*Oct4, Nanog,* and *Klf4*) and three germ layer markers *Nestin, Sox1* (ectoderm), *T* (mesoderm), and *Sox17* (endoderm) during differentiation of mouse iPSCs in an EB model at days 0, 2, 4, and 6. **(C)** Quantitative polymerase chain reaction analysis of gene expression changes between iPSCs (*black*) and day-6 EBs (*gray*). The average of multiple experiments is shown. **(D)** Att-EBs (EBs attached to gelatin dishes) express both Sox2 (*green*) and Nestin (*red*) in Wt- and Homo-EBs without obvious difference in Nestin protein content **(E)**. The mesoderm marker α-SMA **(F)** and endoderm marker GATA4 **(G)** were often found in or around the dense center of the attached EBs. Scale bar = 100 μm. **(H)** Heat map depicting relative mRNA expression levels of endoderm-, mesoderm-, and ectoderm-specific genes in Wt- and Homo-EBs (5–6-day old). Some specific genes for three germ layers were enlarged on the *right*. Expression levels were normalized to the Wt-EBs. *Green* shows elevated, *red* indicates reduced, and *yellow* shows unchanged expression.



SUPPLEMENTARY FIG. S4. Effects of all-trans retinoic acid (RA) on neural induction and cell migration in Wt- and Homo-EBs. **(A**, **B)** Cellular migration was evaluated by measuring the distance from the edge of the EBs to the nucleus of the most distant cell at 48 h after seeding in dishes. *Upper photographs* show spontaneously differentiated EBs at day 6; *lower photographs* display RA-treated EBs at day 10. Values are the mean ± SD (**$P < 0.01$). Scale bar = 100 μm. **(C)** Immunofluorescence demonstrating the expression of βШ-tubulin. *Upper photographs* show spontaneously differentiated EBs at day 6; *lower photographs* display RA-treated EBs at day 10. *White arrowheads* heading to βШ-tubulin-negative cells migrated from EBs. Scale bar = 200 μm.

SUPPLEMENTARY TABLE S1. PRIMERS USED IN THIS STUDY

Sox17 F	CACAGCAGAACCCAGATCTGCAC	RT-PCR for *Sox17*
Sox17 R	CATGTGCGGAGACATCAGCGGAG	
Brachyury F	GTTCCTGGTGCTGGCACCCTCTGC	RT-PCR for *Brachyury*
Brachyury R	CAGACCAGAGACTGGGATACTG	
Sox1 F	GGATCTCTGGTCAAGTCGGAG	RT-PCR for *Sox1*
Sox1 R	CTGGCGCTCGGCTCTCCAGAG	
Nestin F	GGACAGGACCAAGAGGAACA	RT-PCR for *Nestin*
Nestin R	TCCCACCTCTGTTGACTTCC	
Klf4 F	GCGAACTCACACAGGCGAGAAACC	RT-PCR for endogenous *Klf4*
Klf4 R	TCGCTTCCTCTTCCTCCGACACA	
Nanog F	CAGGTGTTTGAGGGTAGCTC	RT-PCR for endogenous *Nanog*
Nanog R	CGGTTCATCATGGTACAGTC	
Oct4 F	TCTTTCCACCAGGCCCCCGGCTC	RT-PCR for endogenous *Oct4*
Oct4 R	TGCGGGCGGACATGGGGAGATCC	
β-actin F	GACAACGGCTCCGGCATGTG	RT-PCR for *β-actin*
β-actin R	GGCTGGGGTGTTGAAGGTCTCAA	
Sox17 F	ACCTACACTTACGCTCCAGTC	qRT-PCR for *Sox17*
Sox17 R	GCCGTAGTACAGGTGCAGAG	
Brachyury F	GAACCTCGGATTCACATCGT	qRT-PCR for *Brachyury*
Brachyury R	TTCTTTGGCATCAAGGAAGG	
Nestin F	TTCCTGAGGTCTCCAGAAGC	qRT-PCR for *Nestin*
Nestin R	GCCATCTGCTCCTCTTTCAC	
Oct4 F	CCAATCAGCTTGGGCTAGAG	qRT-PCR for *Oct4*
Oct4 R	CTGGGAAAGGTGTCCCTGTA	
Nanog F	TACCTCAGCCTCCAGCAGAT	qRT-PCR for *Nanog*
Nanog R	GCAATGGATGCTGGGATACT	
Klf4 F	GTGCCCCGACTAACCGTTG	qRT-PCR for *Klf4*
Klf4 R	GTCGTTGAACTCCTCGGTCT	
β-actin F	GTGACGTTGACATCCGTAAAGA	qRT-PCR for *β-actin*
β-actin R	GCCGGACTCATCGTACTCC	

RT-PCR, reverse transcription–polymerase chain reaction; qRT-PCR, quantitative reverse transcription–polymerase chain reaction.

Supplementary Reference

1. Ray WJ and DI Gottlieb. (1996). Regulation of protein abundance in pluripotent cells undergoing commitment to the neural lineage. J Cell Physiol 168:264–275.