Stem Cell Reports, Volume *2*

Supplemental Information

Suppression of the *SOX2* Neural Effector Gene by PRDM1 Promotes Human Germ Cell Fate in Embryonic Stem Cells

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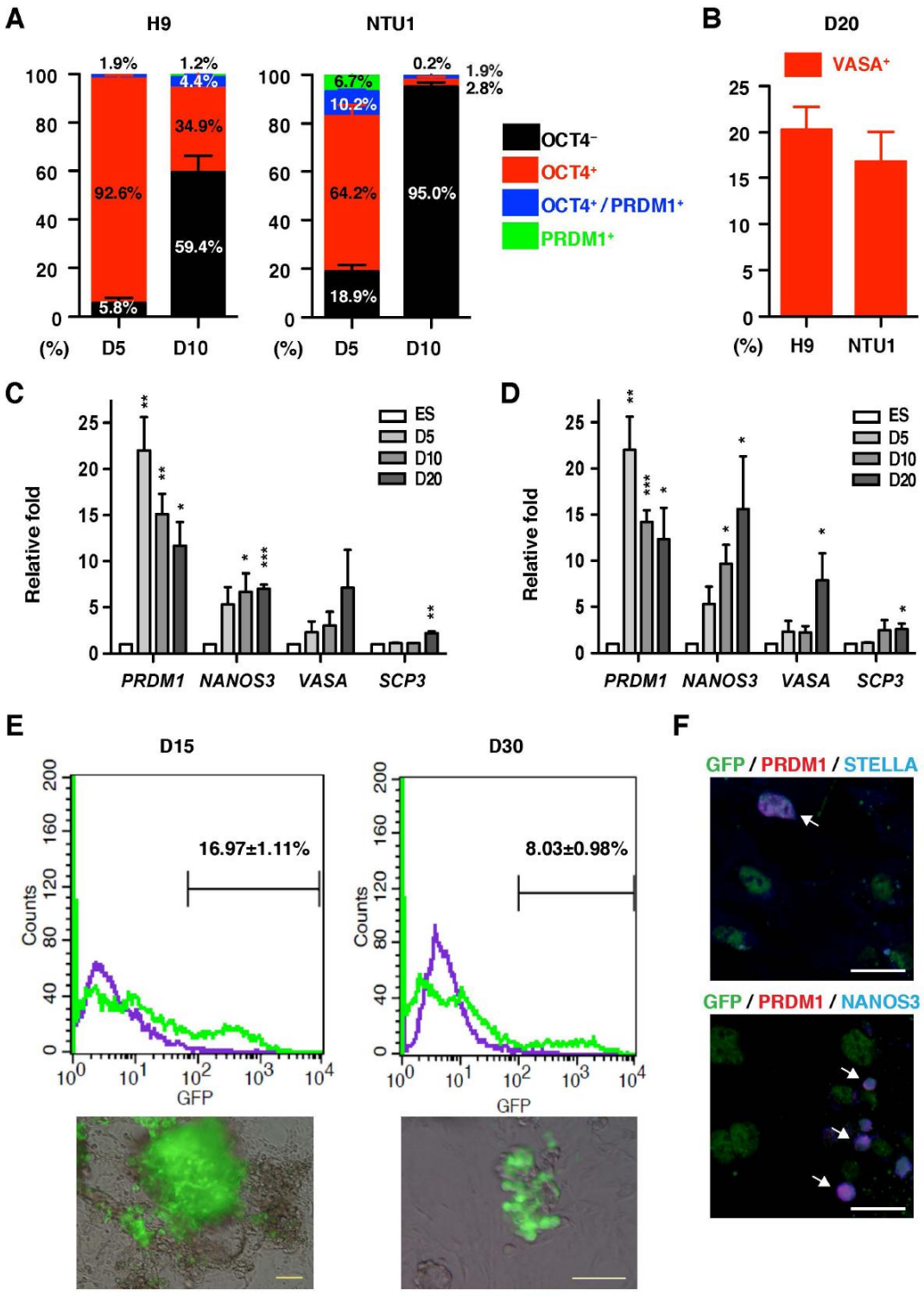
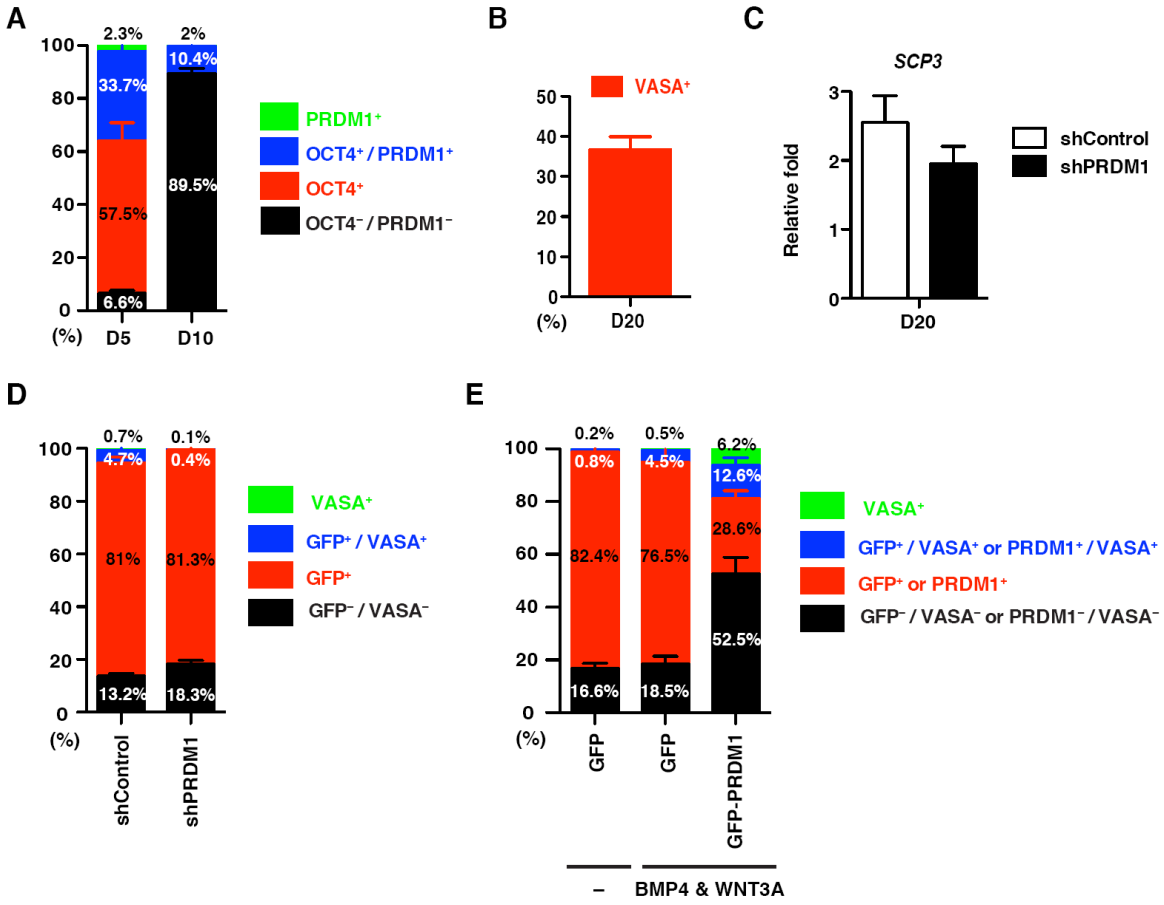
Inventory of Supplemental Information:

Lin et al. Supplemental Figure 1



Lin et al. Supplemental Figure 2



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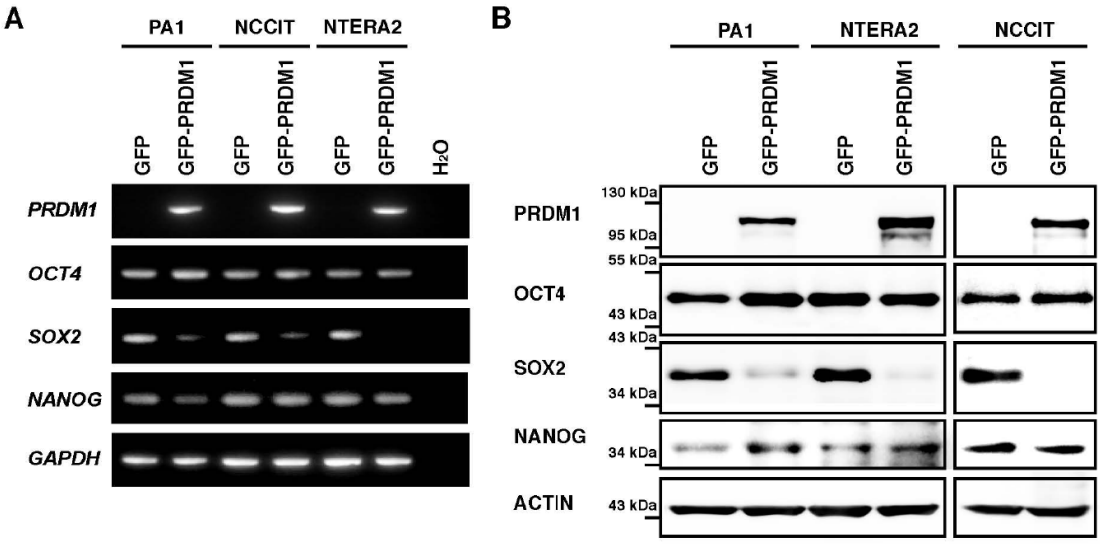
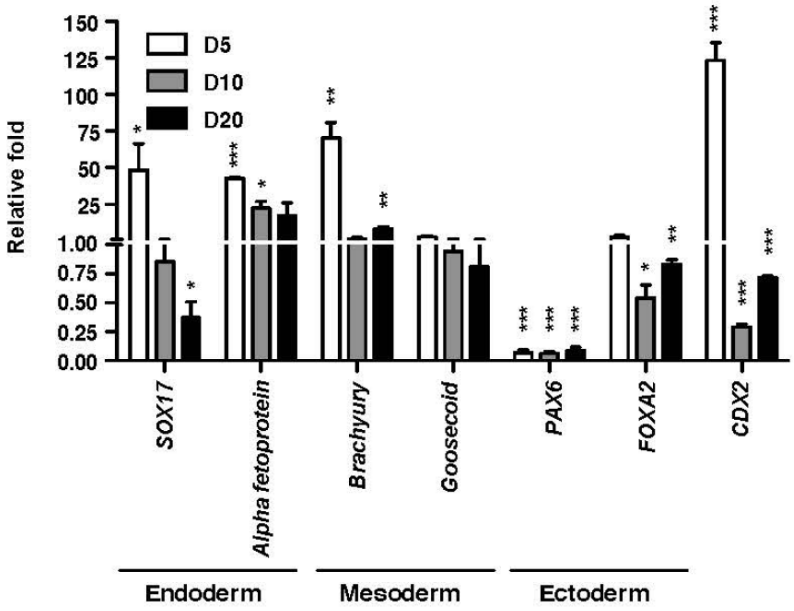
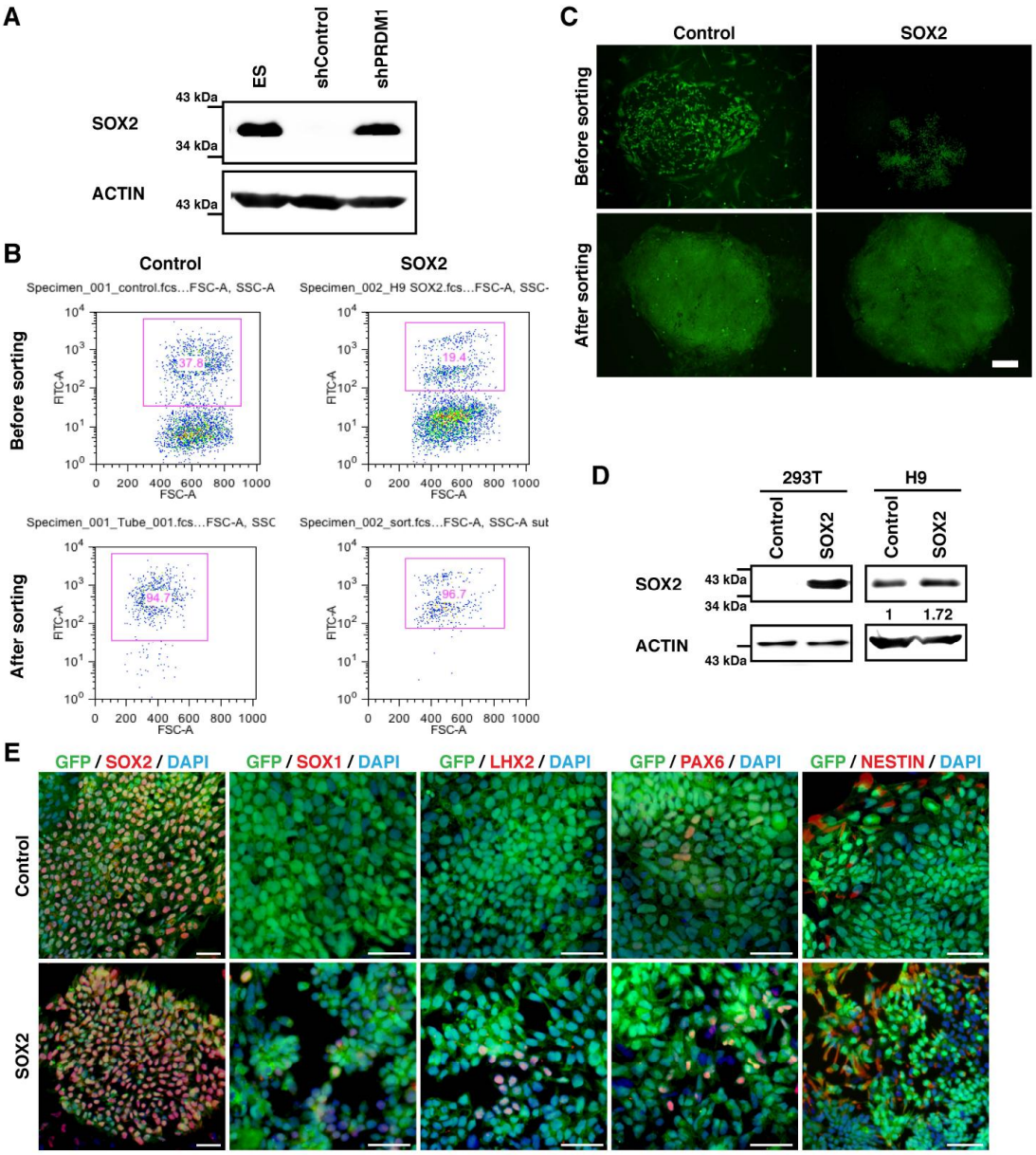


Lin et al. Supplemental Figure 4



Lin et al. Supplemental Figure 5



SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: PRDM1 is expressed in cells derived from spontaneous differentiation of H9 and NTU1 hESCs. (A and B) Frequencies of cells labeled with OCT4 and PRDM1 (A), or VASA (B) were quantified by MetaMorph. H9 and NTU1 hESCs were spontaneously differentiated for indicated days and then immunostained with anti-OCT4, anti-PRDM1 and anti-VASA antibodies. Images of stained cells were automatically counted by using MetaMorph imaging software from 3 independent experiments. (C) RT-QPCR for the indicated mRNA isolated from NTU1 hESCs plated to form embryoid bodies (EBs) in suspension at indicated days. (D) RT-QPCR for the indicated mRNA at indicated days using isolated NTU1 cells that were plated to form EBs for 5 days and switched to adherent culture afterwards. Results (mean ± SEM) in C and D are from 3 independent experiments. (E) FACS analysis of H9: OCT4-EGFP cells spontaneously differentiated for 15 and 30 days. Frequencies of GFP^+ cells (green histogram) were indiated. Results (mean ± SEM) are from 3 independent experiments. Fluorescent images of GFP^+ population of H9: OCT4-EGFP cells at indicated days were shown in the bottom panels. Scale bar, 100 μm. (F) Immunofluorescence staining showed PRDM1 and STELLA or PRDM1 and NANOS3 were co-expressed in some indicated GFP^+ cells acquired from H9: OCT4-EGFP line 10 days after spontaneous differenation. Scale bar, 30 μm.

Supplemental Figure 2: PRDM1 enhanced the effect of BMP4 and WNT3A induced germ cell differentiation. (A and B) H9 hESCs were treated with BMP4 and WNT3A for various days, and immunostained with anti-PRDM1, anti-OCT4 and anti-VASA antibodies. MetaMorph imaging software was used to count the percentage of $PRDM1^+/OCT4^+$ cells at day 5 and day 10 from 2 independent experiments (A) and $VASA^+$ cells at day 20 from 3 independent experiments (B). (C) RT-QPCR analysis showed that knockdown of *PRDM1*

marginally reduced *SCP3* level in H9 hESCs treated with BMP4 and WNT3A for 20 days. Relative expression was normalized with *GAPDH* level and calculated by comparing the mRNA level of *SCP3* to that in shControl group on day 0. Data are represented as the mean ± SEM from 3 independent experiments. (D) Knockdown of PRDM1 reduced the frequency of $GFP^+/VASA^+$ cells in H9 hESCs treated with BMP4 and WNT3A for 10 days, as compared with shControl group. Results were calculated as the percentages of cells with VASA expression in GFP^+ or GFP^- group by MetaMorph from 3 independent experiments. (E) The GFP control or GFP–PRDM1 vector were transduced into H9 hESCs and then differentiated for 12 days in the medium supplemented with or without BMP4 and WNT3A. Results were calculated as the percentages of cells showing VASA expression in GFP^+, GFP^-, $PRDM1^+$ or $PRDM1^-$ group by MetaMorph from 3 independent experiments.

Supplemental Figure 3: Ectopic expression of PRDM1 reduced *SOX2* in EC cells. (A) RT-PCR for the expression of *OCT3/4*, *SOX2*, *NANOG* and *PRDM1* mRNA 2 days after lentiviral transduction with GFP–PRDM1 or GFP encoding vectors (transduction rate between 90~95%) in three indicated human EC lines. *GAPDH* mRNA was used as the internal control. H_2O represents the PCR reaction performed without adding cDNA. (B) Immunoblot analysis showed the reduction of SOX2, but not OCT4 and NANOG, proteins in three human EC lines expressing GFP–PRDM1. Twenty μg of total cell lysates harvested from human EC cells 2 days after transiently transduced with GFP–PRDM1 or GFP expressing vectors was loaded and ACTIN blot was served as the protein loading control.

Supplemental Figure 4: Treatment of H9 hESCs with BMP4 and WNT3A decreased the expression of ectoderm markers. Expression of three germ layer markers, endoderm (*SOX17*, *Alpha fetoprotein*), mesoderm (*Brachyury*, *Goosecoid*), ectoderm (*PAX6*, *FOXA2*),

and trophectoderm (*CDX2*) in H9 hESCs treated with BMP4 and WNT3A at indicated days were examined by RT-QPCR. *GAPDH* was used as the internal control. The data are denoted as means ± SEM relative to hESCs spontaneously differentiated at the indicated days and are from 3 independent experiments.

Supplemental Figure 5: PRDM1 down-regulates SOX2 and establishment /characterization of SOX2-expressing H9 hESCs. (A) Knockdown of PRDM1 restored SOX2 expression in H9 hESCs differentiated with BMP4 and WNT3A. H9 hESCs were transduced with shControl or shPRDM1 vector, followed by induction of BMP4 and WNT3A-mediated differentiation for 3 days. Cell lysates were subjected to immunoblot analysis. ACTIN was used as the internal loading control. (B) Percentage of GFP^+ cells isolated by flow cytometry from H9 hESCs transduced with lentiviral vector encoding control GFP only or SOX2 and GFP simultaneously. (C) Fluorescent images showed the enrichment of GFP-expressing H9 hESCs that were transduced with either control GFP or SOX2 expressing lentiviral vector by cell sorter. Scale bars, 200 μm. (D) Immunoblot analysis was conducted to show the increased SOX2 expression in SOX2 lentiviral vector transdued 293T cells and H9 hESCs as compared to control lentiviral vector transduced cells. ACTIN blot was served as the protein loading control. (E) Immunofluorescence staining showed a higher level of SOX2, SOX1, LHX2, PAX6, and NESTIN expression in SOX2 over-expressing GFP^+ H9 hESCs than in control GFP^+ H9 hESCs. Scale bars, 50 μm.

SUPPLEMENTAL TABLES

Supplemental Table3

Table S3: Average expression correlation coefficients between GFP-transduced and GFP-PRDM1 transduced H9 cells and various tissues including fetal gonads and testis substructures.

Tissue/Cell type	GFP-transduced H9	GFP-PRDM1-transduced H9
Avg over 158 experiments of differentiated tissues	0.1844	0.193
Fetal ovary	0.275	0.3659
Fetal testis	0.285	0.3738
hESCs	0.6435	0.3474
hiPSCs	0.6338	0.3456
Adult testis substructures	**GFP-transduced H9**	**GFP-PRDM1-transduced H9**
Germ cell	0.148	0.176
Leydig cell	0.114	0.138
Seminiferous tubule	0.13	0.131
Interstitial cell	0.106	0.119

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Human EC culture

Human embryonal carcinoma cell lines PA-1 (ovarian teratocarcinoma), NTERA-2 cl.D1 (NT2) (pluripotent testicular embryonal carcinoma) and NCCIT (teratocarcinoma) were obtained from Bioresource Collection and Research Center, Taiwan (BCRC). PA-1 cells were maintained in MEM (Invitrogen) supplemented with 10% FBS (Biological industries), 0.1 mM non-essential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate. NTERA-2 cl.D1 was grown in DMEM medium (Invitrogen) supplemented with 10% FBS, 4 mM L-glutamine and 4.5 g/L glucose. NCCIT was maintained in RPMI 1640 medium (Invitrogen) plus 10% FBS.

Immunohistochemistry

The paraffinized sections were subjected to the treatment with antigen retrieval reagent in Tris/EDTA (pH 9) solution (Dako). Sections were incubated with 0.1% Triton X-100 in PBST for 20 min and were then blocked with 3% human serum (Invitrogen) for 30 min. Sections were next incubated overnight with primary antibody or control antibody at 4°C, followed by incubation with enzyme-conjugated secondary antibody at room temperature for 1 hr. The color development was carried out by incubation with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) (Roche) or 3, 3′-diaminobenzidine (DAB) (Invitrogen) chromogen, depending on either alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibody used. The sections were washed in tap water for 5 min, dehydrated, and mounted with cover slips. The primary antibodies used in this study are: mouse anti-PRDM1 antibody (Chang et al., 2002), rabbit anti-OCT4 antibody (Santa Cruz), goat anti-VASA antibody (R&D System) and rabbit anti-VASA antibody (Abcam). For double staining, the sections were incubated with the

secondary primary antibody for overnight at 4°C after the color development of first staining. Results of immunohistochemistry staining in this study are representative of at least 3 independent experiments.

Immunofluorescence staining

hESCs were rinsed with PBS twice and fixed in 4% paraformaldehyde at room temperature for 20 min, followed by permeabilization with 0.1% Triton X-100 for 5 min at room temperature. Cells were then blocked by 1% bovine serum albumin in PBS for 1 hour at room temperature. Primary antibodies in blocking buffer were added for overnight incubation at 4°C. Cells were washed three times with PBS and then incubated with secondary antibodies for 1 hour at room temperature. Cell nuclei were counterstained with DAPI (Sigma) in PBS. The fluorescent images were examined by either a confocal microscope (Leica SP5) or a fluorescence microscope (Leica DM6000B). The intensity of fluorescence signals was enhanced by a commercial fluorescein-labeled tyramide signal amplification kit (Invitrogen). Antibodies used in this study are: mouse anti-PRDM1 antibody (Chang et al., 2002), rabbit anti-PRDM1 antibody (Cell Signaling), rabbit anti-OCT4 antibody (Santa Cruz), rabbit anti-SOX2 antibody (Abcam), mouse anti-Lim1/2 (LHX2) antibody (Developmental Studies Hybridoma Bank), rabbit anti-SOX1 antibody (Chemicon), goat anti-GFP antibody (Santa Cruz), mouse anti-GFP antibody (Abcam), rabbit anti-NANOG antibody (Abcam), rabbit anti-NANOS3 antibody (Abcam), rabbit anti-STELLA antibody (Abcam), goat anti-VASA antibody (R&D Systems), mouse anti-NESTIN antibody (Chemicon) and mouse anti-PAX6 antibody (Developmental Studies Hybridoma Bank), mouse anti-TUJ1 antibody (Chemicon) and rabbit anti-MAP2 antibody (Millipore). The secondary antibodies used are: anti-rabbit IgG conjugated with Alexa Fluor 350 dye (Invitrogen), anti-mouse IgG conjugated with Alexa Fluor 488 dye (Invitrogen), anti-goat IgG conjugated with Alexa Fluor 488 dye

(Invitrogen), anti-mouse IgG conjugated with Alexa Fluor 555 dye (Invitrogen), anti-rabbit IgG conjugated with Alexa Fluor 555 dye (Invitrogen) or anti-goat IgG conjugated with Alexa Fluor 555 dye (Invitrogen). Results of immunofluorescence staining shown in this study are representative of at least 3 independent experiments. To calculate the percentage of cells showing positive fluorescent signal, cell images were photographed and analyzed by MetaMorph (Molecular Devices). Three randomly selected fields (20 × magnification) that contained more than 200 cells in each field were counted. The total number of cells was estimated by counting the DAPI-labeled nuclei.

Meiotic spreads and meiotic marker staining

Cells were suspended with hypo-extraction buffer (pH 8.2) for 30 min and then mixed with 100 mM sucrose and dropped on glass slides coated with 1% paraformaldehyde and 0.15% Triton X-100 in PBS (pH 9.2). Slides were incubated at room temperature until the suspension was dried and blocked with 4 % goat serum in TBST for 40 min. Primary antibody, rabbit anti-SCP3 antibody (1:1000, Abcam), was applied overnight at 4°C. Slides were then washed with TBST and incubated for 60 min at room temperature with goat anti-rabbit secondary antibody labeled with Alexa Fluor 555 dye. DAPI was stained as a nuclear indicator. Slides were mounted with Fluorescence Mounting Medium (Dako). Results shown in this study are representative of at least 3 independent experiments.

Fluorescent in situ hybridization (FISH)

Single cell suspension prepared in Carnoy's fixative (1: 3 [v/v], acetic acid: methanol) was placed on clean microscope slides and dried in air at room temperature. Chromosome Enumeration Probes (Abbott Molecular) were used for hybridization according to the manufacturer's instruction. Briefly, slides were incubated in denaturation buffer for 5 min at

73°C, and dehydrated sequentially in 70% EtOH, 85% EtOH and 100% EtOH for 1 min each. A mixture of CEP hybridization buffer and probes composing of CEP 16 SpectrumGreen and CEP X SpectrumOrange were applied onto slides at 50°C, and then sealed with coverslips immediately. Slides were placed in a humidified chamber overnight at 42°C, followed by washing with 0.4 × SSC buffer added with 0.3% NP-40 for 2 min at 73°C, and with 2 × SSC buffer added with 0.1% NP-40 for 1 min. Slides were then dried in darkness and mounted with counterstain DAPI II. Images were captured under the Olympus BX-61 fluorescence microscope. Results shown in this study are representative of at least 3 independent experiments.

Reverse transcriptase (RT) –PCR or RT –QPCR

Total RNA isolation, cDNA synthesis, and subsequent RT-QPCR analysis in an ABI Prism 7300 sequence detection system or RT-PCR analysis was according to a published protocol (Su et al., 2009), by using an RNeasy spin column (Qiagen) for RNA isolation and High Capacity cDNA Reverse Transcription kit (ABI) for cDNA synthesis. RT-PCR or RT-QPCR analysis of the cDNA was carried out using the primer sets or the primers for the SYBR Green method. Primers sequences are: *GAPDH*: 5'-CGGGAAACTGTGGCGTGATG-3' and 5'-TGGAGGAGTGGGTGTCGCTGTT-3', *PRDM1*: 5'-CGAAATGCCCTTCTACCCTG-3' and 5'-GCGTTCAAGTAAGCGTAGGAGT-3', *OCT3/4*: 5'-CTTGCTGCAGAAGTGGGTGGAGGAA-3' and 5'-CTGCAGTGTGGGTTTCGGGCA-3', *SOX2*: 5'-CCGTTCATCGACGAGGCTAA-3' and 5'-CCTTCTTCATGAGCGTCTTGGT-3', *NANOG*: 5'-TGGGCCTGAAGAAAACTATCCA-3' and 5'-GAGGAAGGAAGAGGAGAGACAGTCT-3', *NANOS3*: 5'-GACGCTTCTGCCCACTTACTG-3' and 5'-CTTCTTGCCTGCCGAGTTTC-3', *VASA*:

5’-CCAAGAGAGGCGGCTATCG-3’ and 5’-CACGGCAACCTCGGAAACT-3’, *DAZL*: 5’-GCCAGCACCCAGTCCTCAT-3’ and 5’-AACATCAATTCCTCCAACAAAAACA-3’, *SSEA1*: 5’-CGCTGGGTTTGGATGAACTT-3’ and 5’-GGTAGAGGTAGCCATAAGGCACAA-3’, *SCP3*: 5’-TGCCAAGAGAAAGAGACTAGAAATGTAT-3’ and 5’-CTGCCTTTGATCTTGTTGTGTTTT-3’, *DNMT3b*: 5’-TGGAGGCAGACAGTGGAGATG-3’ and 5’-AGGTCCCCTATTCCAAACTCCTT-3’. Other lineage markers: *SOX17*: 5’-TTCGTGTGCAAGCCTGAGAT-3’ and 5’-GTGTGTAACACTGCTTCTGGCC-3’, *Alpha fetoprotein:* 5’-GCAGAGGAGACGTGTTGGAT-3’ and 5’-CCAGTGTGGTCAGTTTGCAG-3’, *Brachyury* : 5’-CCAATGAGATGATCGTGACCA-3’ and 5’-ATTCCCCGTTCACGTACTTCC-3’, *Goosecoid*: 5’-CGGAGAAGTGGAACAAGACGT-3’ and 5’-GCGTGTGCAAGAAAGTAGCATC-3’, *FOXA*2: 5’-GACGCTGAGCGAGATCTACCA-3’ and 5’-CGAGTGGCGGATGGAGTT-3’, *PAX6*: 5’-TCAGCACCAGTGTCTACCAACCAA-3’ and 5’-ATCATAACTCCGCCCATTCACCGA-3’, *SOX1*: 5’-TACAGCCCCATCTCCAACTC-3’ and 5’-GACTTCACCAGAGAGCCCAG-3’, *LHX2*: 5’-GTCCTCCAGGTCTGGTTCCA-3’ and 5’-CCGTGTTTTCCTGCCGTAA-3’, *NESTIN*: 5’-GCCACCTCACTGCAGTAGTG-3’ and 5’-AGTCAGCACATGGGAGTGC-3’, *MAP2*: 5’-GCTCTGGCTCCCAGTGTATTTAA-3’ and 5’-CCTGTAAAGCAGGAATCTTTGACA-3’, *CDX2*: 5’-CCAAGTGAAAACCAGGACGAA-3’ and 5’-GATGGTGATGTAGCGACTGTAGTGA-3’. *GAPDH* was used as the internal control for normalization in this study.

Immunoblotting

Total cells lysates isolated from hEC, 293T and H9 hES cells were lysated by 2 × SDS sample buffer and separated by 10% SDS-PAGE. The subsequent immunoblotting was performed according to a published protocol (Su et al., 2009). Mouse anti-PRDM1 (1: 2,000 dilution) (Chang et al., 2002), rabbit anti-SOX2 (1: 1,000 dilution, Abcam), mouse anti-OCT4 (1: 1,000 dilution, Santa Cruz), rabbit anti-NANOG (1: 1,000 dilution, Abcam) and mouse anti-β-ACTIN (Sigma) antibodies were used. Horseradish peroxidase–conjugated secondary antibodies (Sigma) were used. Chemiluminescent signals were detected by LAS-3000 (Fujifilm).

Generation of lentiviral vector and transduction

To introduce a marker for tracing transduced cells, a DNA fragment containing ubiquitin-C promoter driven-green fluorescent protein cDNA (UbC-GFP) was cut from pFUGW plasmid (Lin et al., 2007; Lin and Calame, 2004), by PacI and EcoRI digestion. The UbC-GFP fragment was inserted into pSin-EF2-SOX2-Pur (Addgene) vector and pSin-EF2-Pur vector by blunt end ligation at Bsu36I site. The procedure for preparing lentiviral vectors and transduction was performed as previously described (Lin et al., 2007; Lin and Calame, 2004). 293T and 3T3 cells were maintained as described previously (Lin et al., 2007; Lin and Calame, 2004). Lentiviral vectors expressing shRNA for human PRDM1 or GFP−PRDM1 fusion protein were described previously (Lin et al., 2007; Tsai et al., 2008). Human H9 hESCs were transduced with virus at a multiplicity of 10 in the presence of 5 μg/mL of polybrene. Lentiviral vectors producing shRNAs targeting PRDM1 were transduced to H9 hESCs every 5 days during differentiation. GFP^+ cells were enriched manually via fluorescence microscopy. In some experiments, GFP^+ transduced cells were sorted by FACS Aria (BD Biosciences) prior to further analysis.

Chromatin immunoprecipitation (ChIP)

A total of 1×10^7 lentivirally transduced NCCIT cells were fixed with formaldehyde to a final concentration of 1% at room temperature for 10 min. Cells were then collected and resuspended with lysis buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 and 0.25% Triton X-100), the subsequent sonication and precipitation was performed essentially according to a previous report (Su et al., 2009). Five μg of anti-PRDM1 antibody (Chang et al., 2002) and Dynal magnetic beads conjugated with protein A (Invitrogen) were used here to pull down the protein-chromatin complexes. DNA was finally purified by phenol/chloroform extraction and then used for PCR analysis. Primer sequences used for PCR were *SOX2* site 1&2: 5'-TTCTCCAGTTGCTGCACTTAGC-3' and 5'-CCTAACCTTCCCACAATCTCCTCTA-3', *SOX2* site 3: 5'-AAGTTGTTATATATCCTCCCAAGGAAAA-3' and 5'-TTTGAAGAAACAACCAAGGAATTG-3', *SOX2* site 4: 5'-CTACAGTCCCCTGTCTCCACAAA-3' and 5'-GCCCTAGCCAAAAGGCCTTA-3', *SOX2* site 5: 5'-CGCCTTCTCTTCCCCAGATC-3' and 5'-CCGGAAAGGGTGAGATTGG-3', *SOX2* site 6: 5'-CGCCGAATATGTATGCATTTTG-3' and 5'-CCCGAAGGTTCTCCTTTTTCTC-3', *SOX2* site 7: 5'-TTTTAGCCACAAAGATCCCAACA-3' and 5'-AATGGCAGCACGTCCGTTAC-3' and *SOX2* 3'UTR: 5'-ACCCAGTCACCGTGGTAACTG-3' and 5'-AGATCTGGCCTGACACTCCAA-3'. Values obtained from immunoprecipitated samples were normalized to that of their corresponding input samples.

Transfection and luciferase reporter assay

Human *SOX2* gene fragment containing −4437 to +268 was amplified from genomic DNA of human peripheral blood cells, and cloned into pGL3 basic (pGL3B) vector (Promega). The details of construction of *SOX2*–Luc and its various deletion mutants or PRDM1 binding site mutants will be available upon request. Various *SOX2*–Luc constructs (600 ng) and 1.25 μg of PRDM1 expression vector (Lin et al., 1997) or control vector, and 4 ng of Renilla luciferase reporter driven by the thymidine kinase (tk) promoter (RL-tk) were co-transfected into NCCIT cells by polyethylenimine (PEI, Sigma) method. After 48 hr, cells were lysed and subjected to firefly luciferase and Renilla luciferase assays as described previously using the TopCount NXT™ instrument (Su et al., 2009). Relative repression fold was calculated according to previous reports (Su et al., 2009; Ying et al., 2012).

Microarray

Total RNA was prepared from H9 hESCs transduced with GFP or GFP−PRDM1 lentiviral vector for 10 days and 17 days in ES media and then used for Affymetrix GeneChip microarray analysis with the sequential procedures of reverse transcription, biotin-conjugated nucleotide incorporation/fragmentation and converting linear RNA into 35–200 nt biotin-labeled fragments by using GeneChip® 3' IVT Express Kit. Samples were then hybridized to GeneChip® Human Genome U133 array. The following steps for washing and staining arrays were preformed by using Fluidics Station 450, and the array data were acquired with GeneChip® Scanner 3000.

Comparison with external gene expression microarray data and Gene Ontology (GO) analysis

For comparing the gene expression profiles of hESCs transduced with GFP−PRDM1 and GFP control vectors with other tissues, we used three external datasets: (1) 6 experiments of

hESCs and hiPSCs derived from skin (GEO id GSE27280, (Huang et al., 2011)), (2) 34 experiments of human fetal testis and ovary tissues (GEO id GSE15431, (Houmard et al., 2009)), (3) 158 experiments of differentiated tissues of human adults (GEO id GSE1133, (Su et al., 2004)). The following procedures were undertaken in comparative analysis according to a previous report (Yeang, 2010). First, we rank-transformed the hybridization levels reported in the microarray data into the cumulative distribution function (CDF) values, so that each value was scaled between 0 and 1. Second, for each gene and experiment we took the average over the values of multiple probes and used this number to represent the gene expression level. Third, we calculated the log2 ratios of gene expressions between the hES cells expressing GFP−PRDM1 and GFP control and computed the distribution of log2 ratios accordingly. The genes below 5 percentile or above 95 percentile of the distribution were selected as differentially expressed between GFP−PRDM1 and control transductions. We then took the union of these two gene sets and intersected them with the genes appeared in the three external datasets. 1,071 genes passed these filtering criteria. Fourth, we evaluated the correlation coefficients between the expression profiles of all experiments (including the external datasets) restricted to the 1,071 selected genes. Fifth, based on these correlation coefficients we applied a Matlab function of non-classical multi-dimensional scaling (MDS) to project the data points into a two-dimensional plane. MDS projects high-dimensional data points into a low-dimensional space by minimizing the distances between the points. Axes of the projected two-dimensional plane are arbitrary and do not indicate any specific genes. However, distances between projected data points approximate dissimilarity (1 – correlation coefficient) of the corresponding expression profiles. The average gene expression profile over the 158 differentiated tissues from the GSE1133 dataset was treated as a reference point in the multi-dimensional scaling plot. BiNGO (Maere et al., 2005), a plugin of the Cytoscape, was used to determine the Gene Ontology (GO) of categories related to biological process of

1,071 genes that show statistic significance ($p < 0.0001$).

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