Methods

Animals care and use. Specific-pathogen-free-grade mice were obtained from Beijing Vital River Laboratories and housed in the animal facilities of the Chinese Academy of Sciences. All the studies were carried out in accordance with the Guidelines for the Use of Animals in Research issued by the Kunming Institute of Zoology, Chinese Academy of Sciences. Female mice of B6D2F1 (C57BL/6J × DBA/2)-ROSA26–CAG-tdTomato were used to provide oocytes, female mice of FVB were used for mating with male mice of B6D2F1 to provide zygotes, and Female mice of ICR were used as pseudopregnant surrogates for micromanipulation.

Oocytes collection. Mature oocytes were collected from eight-week-old female mice that had been induced to superovulate by injection of 5.0-7.5 IU pregnant mare's serum gonadotropin(PMSG) followed by 5.0-7.5 IU human chorionic gonadotropin(hCG). Oocytes were collected from the oviduct 13-15 hours after hCG injection, and were placed in HEPES-CZB and then treated with 0.1% hyaluronidase until the cumulus cells dispersed. The derived oocytes were cultured in KSOM with paraffin oil (Sigma) and stored at 37℃ (5% CO2/air).

Zygotes collection. Eight-week-old female mice that had been induced to superovulate by injection of 5.0-7.5 IU PMSG followed by 5.0-7.5 IU hCG were used. Then female mice were mated with male mice. Zygotes were collected from the oviduct 19-20 hours after hCG injection, and were placed in HEPES-CZB and then treated with 0.1% hyaluronidase until the cumulus cells dispersed. The derived oocytes were cultured in KSOM with

paraffin oil (Sigma) and stored at 37℃ (5% CO2/air).

Derivation of PG-DhESCs. The PG-DhESCs was derived as previously described1. Mature oocytes were obtained from superovulated B6D2F1-ROSA26-CAG-tdTomato female mice, and then oocytes were activated for 5–6 h in the activation medium containing 10 mmol/L SrCl2. Following activation, all the activated embryos were cultured in the KSOM medium at 37 °C under 5% CO2 /air. The activated embryos that reached the morula stage or blastocyst stage by 4.5 days were used for the derivation of ESCs. The zona pellucida was removed using acid Tyrode solution. Each blastocyst and morula was transferred into one well of a 96-well plate seeded with ICR embryonic fibroblast feeders in different ESC media. The culture medium was DMEM, 100× NEAA, 100× Nucleosides, 100× L-Glutamine, 100× P/S, 100× β-mercaptoethanol, 15% FBS and 1000 U/mL LIF, which was supplemented with 1 μmol/L PD0325901 (Selleck) and 3 μmol/L CHIR99021 (Selleck).

Immunofluorescence. Cell samples were fixed with 4% paraformaldehyde for 30 min at room temperature and then washed three times with PBS. The cells were permeabilized in 0.1% Triton X-100 (Solarbio) in PBS for 30 min at RT. Subsequently, blocking was performed using 4% bovine serum albumin (BSA) for 1h at room temperature. The samples were then incubated with primary antibodies overnight at 4℃. Primary antibodies included anti-OCT4 (Zen) and anti-NANOG (Santa Cruz). Following incubation, samples were washed three times with PBST, incubated with GFP secondary antibody (Invitrogen) and CY5 secondary antibody (Invitrogen) at room temperature for 2 h, and then imaged

with a LSM780 Meta confocal microscope (Zeiss).

RNA extraction and Real-time quantitative PCR. Total RNA was isolated from the cells using Trizol reagent (Tiangen). The cDNA was obtained from about 400 ng RNA with a reverse transcription reaction by the FastKing RT Kit (Tiangen). Real-time quantitative PCR reactions (Tiangen) were performed on a Bio-Rad CFX96 using the SYBR Green Mix (Tiangen). All the gene expression levels were normalized to the internal standard gene Gapdh. The primer sequences are listed in Supplemental Table 3.

Bisulfite PCR. The BSP of this research was operated by Sangon Biotech (Shanghai, China) and Tsingke Biotechnology (Beijing, China). In brief, treatment of genomic DNA with bisulfite converts all unmethylated cytosines to uracil, whereas methylated cytosines remain unchanged;" Then primers were designed at both ends of the CpG island for PCR amplification, and the target product was purified and TA cloned. Positive clones were selected from each clone for sequencing. Finally, the measured sequence was compared with the original sequence, the number and degree of methylation were counted.

CRISPR/Cas9 and pronuclear injection. The methods of gene knockout were performed as previously described2. Two sgRNAs targeting distinct positions of coding exons of Prdm14 were designed using the sgRNA design tool (http://crispor.gi.ucsc.edu/). Sequences complementary to the sgRNAs were cloned into the px458 vector by oligo annealing. The T7 promoter was added to sgRNA templates by PCR using the primers listed in (Supplemental Table 3), then sgRNAs were synthesized by in vitro transcription (IVT) from the templates using T7 high yield RNA transcription kits (Vazyme). Synthesized

sgRNAs were purified using EasyPure RNA purification kits (Transgen). The sgRNAs and Cas9 protein (IDT) were adjusted to 500 ng/μl with ultrapure water and aliquots were frozen at -80°C until use. A mixture of the two sgRNAs (25 ng/μl each) targeting Prdm14 and Cas9 protein (50 ng/μl) was injected into zygotes using a micromanipulator (Eppendorf) at 5–6 h after insemination. The injected embryos were cultured as above in KSOM for 24 h until they reached the 2-cell stage or for 48 h until they reached the 4-cell stage.

Generation of chimeric mice and embryo transfer. PG-DhESCs were injected into FVB × B6D2F1 8-Cell embryos, which had been treated with Prdm14-sgRNA and Cas9 protein at the zygote stage. 8-10 cells were injected into each embryo. About 1 h after injection, the embryos were transferred to the oviducts of E0.5 pseudopregnant ICR female mice. For the recovery of full-term pups, recipients were euthanized and subjected to Caesarean section at E18.5. Living pups were nursed by lactating ICR females. The systemic contribution of PG-DhESC in chimeric mice was estimated from their coat color.

RRBS and data analysis. RRBS was performed by Novogene (Tianjin, China). In addition to the RRBS data produced in this experiment, other RRBS datasets, including WT oocyte and sperm3 , and PGC at different embryonic days (E10.5, E13.5, and E16.5)4 were downloaded from GSE56697 and the Nodai genome database (http://www.nodai-genome.org/ mouse.html?lang=en). All these data were analyzed together5. Briefly, the sequencing reads were mapped to the mouse genome (version mm10) by Bismark v0.22.3 (Babraham Bioinformatics) after trimming by Trim Galore

v0.4.5 (Babraham Bioinformatics) with the‘‘-rrbs’’ option. The methylation levels of covered cytosine sites were calculated by dividing the number of reported C with the total number of reported C and T. CpG sites covered by more than ten reads were used for the analysis. Heatmap shown in the manuscript were plotted with Matplotlib of Python. Histograms of methylation level distribution were drawn by the ggplot2 package of in R. The methylation levels of the CpG sites covered in all the samples were used in the analyses.

RNA-seq and data analysis. RNA-seq was performed by Novogene (Tianjin, China). RNA-seq data from ESCs were obtained from the European Nucleotide Archive (accession number: GSE99494)6. After the RNA sequencing data were obtained, low-quality reads with quality less than 20 and length less than 36 were removed using TRIMGALORE software. Eligible reads were mapped into the mouse reference genome of GRCm39 with STAR software and the raw counts of gene expression of each sample were obtained with featurecounts. The raw count numbers of genes from different sample were converted into TPM using a self-written Perl script to compare the differences between groups, and the significance analysis of differences was completed by DEseq2 software.

Genotyping. Genomic DNA from blastocysts was extracted using the TIANamp genomic DNA kit (Tiangen) following the manufacturer’s instructions. PCR was performed using 2× Rapid taq master mix (Vazyme) with gene-specific primers.The primers were used as listed in Supplemental Table 3. The amplified products were purified by gel

electrophoresis using a DNA Purification Kit (Omega) and cloned into 5 min TA/Blunt-Zero Cloning Kit (Vazyme). For each sample, more than 10 to 20 coli-clones were picked for sequencing.

Analysis of off-target sites. The 4 potential off-target sites were predicted by the sgRNA design tool (http://crispor.gi.ucsc.edu/). The primers used for PCR are listed in Supplemental Table 3. PCR products were sequenced by the Sanger sequencing for site1, site2, site3 and site4, respectively.

Statistic Analysis. Statistical analysis was performed using PRISM10 statistical software. The Student's t test was used for statistical analysis. For all statistical analyses, a value of $p < 0.05$ was considered to be statistically significant.

Reference

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