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Differential gene expression in mouse spermatogonial stem cells and embryonic stem cells

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Abstract. Mouse spermatogonial stem cells (mSSCs) may be reprogrammed to become pluripotent stem cells under *in vitro* culture conditions, due to epigenetic modifications, which are closely associated with the expression of transcription factors and epigenetic factors. Thus, this study was conducted to compare the gene expression of transcription factors and epigenetic factors in mSSCs and mouse embryonic stem cells (mESCs). Firstly, the freshly isolated mSSCs [mSSCs (f)] were enriched by magnetic-activated cell sorting with Thy1.2 (CD90.2) microbeads, and the typical morphological characteristics were maintained under *in vitro* culture conditions for over 5 months to form long-term propagated mSSCs [mSSCs (l)]. These mSSCs (l) expressed pluripotency-associated genes and were induced to differentiate into sperm. Our findings indicated that the mSSCs (l) expressed high levels of the transcription factors, *Lin28* and *Prmt5,* and the epigenetic factors, *Tet3*, *Parp1*, *Max*, *Tert* and *Trf1,* in comparison with the mESCs, with the levels of *Prmt5*, *Tet3*, *Parp1* and *Tert* significantly higher than those in the mESCs. There was no significant difference in Kdm2b expression between mSSCs (l) and mESCs. Furthermore, the gene expression of *N-Myc*, *Dppa2*, *Tbx3*, *Nr5a2*, *Prmt5*, *Tet3*, *Parp1*, *Max*, *Tert* and *Trf1* in the mSSCs (l) was markedly higher in comparison to that in the mSSCs (f). Collectively, our results suggest that the mSSCs and the mESCs displayed differential gene expression profiles, and the mSSCs possessed the potential to acquire pluripotency based on the high expression of transcription factors and epigenetic factors. These data may provide novel insights into the reprogramming mechanism of mSSCs.

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Abbreviations: *Dnmt1*, DNA methyltransferase 1; *Dmrt1*, doublesex and mab-3 related transcription factor 1; *Dot1l*, disruptor of telomeric silencing 1-like; *Dppa2*, developmental pluripotency associated 2; *Esrrb*, estrogen-related receptor b; iPSCs, induced pluripotent stem cells; *Kdm2b*, lysine (K)-specific demethylase 2b; *Klf2*, Krüppel-like factor 2; *Klf4*, Krüppel-like factor 4; *Lin28*, Lin-28 homolog A; MACS, magnetic-activated cell sorting; *Max*, Myc associated factor x; MEF, mouse embryonic fibroblast; mESCs, mouse embryonic stem cells; mSSCs (f), freshly isolated mouse spermatogonial stem cells; mSSCs (l), long-term propagated mouse spermatogonial stem cells; *Nr5a2*, nuclear receptor subfamily 5, group A, member 2; *Oct4*, octamer-binding transcription factor 4; *Parp1*, poly[ADP-ribose] polymerase 1; *Prdm14*, PR domain containing 14; *Prmt5*, protein Arg N-methyltransferase 5; RS, round spermatid; *Sox2*, Sry (sex determining region Y)-box 2; SSCs, spermatogonial stem cells; *Tbx3*, T-box 3; *Tert*, telomerase reverse transcriptase; *Tet1*, ten-eleven translocation methylcytosine dioxygenase 1; *Tet2*, ten-eleven translocation methylcytosine dioxygenase 2; *Tet3*, ten-eleven translocation methylcytosine dioxygenase 3; *Trf1*, telomeric repeat binding factor 1; *Utf1*, undifferentiated embryonic cell transcription factor 1; *Zscan4c*, zinc finger and SCAN domain containing 4c.

Key words: mouse spermatogonial stem cells, mouse embryonic stem cells, transcription factors, epigenetic factors, gene expression, reprogramming

Introduction

Spermatogonial stem cells (SSCs) are unipotent germ cells which have been demonstrated to express many pluripotency-associated genes as well as alkaline phosphatase (AP) activity as they are pluripotent stem cells (PSCs) (1,2). They also possess the potential ability to reacquire pluripotency due to spontaneous epigenetic reprogramming (3). Epigenetic mechanisms are closely associated with the induction and the maintenance of pluripotency (4). Previous findings have revealed the complex connection between epigenetic modification factors and pluripotent transcription factors, both of which control gene expression directly linked to pluripotency and reprogramming (5). It has been demonstrated that the

generation of induced (i)PSCs relies on the exogenous expression of transcription factors (such as *Oct4*, *Sox2*, *N-Myc* and *Klf4)*, which is an inefficient and random reprogramming process (6). However, epigenetic factors have been shown to provide a more powerful means of improving reprogramming efficiency (7). In fact, the molecular mechanism responsible for the *in vitro* reprogramming of SSCs may provide insight into the epigenetic reprogramming of iPSCs (5).

Although previous experiments have investigated the differences in transcript and proteomic profiles between mouse (m)SSCs and mouse embryonic stem cells (mESCs) (8,9), differences in the expression of crucial transcription factors and epigenetic factors remain unclear. A recent study has indicated that the loss of *Dmrt1*, *Dnmt1* and tumor protein (*Tp)53* expression, and the overexpression of *Oct4* increased the rate of mSSC reprogramming (10). However, the mechanism of SSC reprogramming to PSCs remains unknown, particularly due to the difficulty of tracing orchestrated epigenetic changes during the very low-efficiency reprogramming process (10). As a result, it becomes increasingly important to determine the differential gene expression of pluripotent factors and epigenetic factors in mSSCs and mESCs in order to elucidate the mechanism of mSSC reprogramming. Thus, we examined the relative mRNA expression of ESC-associated transcription factors and epigenetic factors in freshly isolated mSSCs [mSSCs (f)] and long-term propagated mSSCs [mSSC (l)] versus mESCs.

Materials and methods

Isolation of mSSCs (f). The mSSCs were isolated from 6-day-old imprinting control region (ICR) male mouse testes at our laboratory by two-step enzyme digestion and magnetic-activated cell sorting (MACS) with CD90.2 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (11). The experiment was repeated >3 times and 30 mice were used each time. The mice were sacrificed by decapitation and the testes were removed for the isolation of mSSCs. All procedures were performed in accordance with the animal care guidelines of the Institutional Animal Care and Use Committee of Guangzhou Medical University (Guangdong, China) and were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Culture of mSSCs and mESCs. The purified mSSCs (f) were cultured on mouse embryonic fibroblast (MEF) feeder cells treated with mitomycin C (Sigma, St. Louis, MO, USA). The cells were cultured in StemPro-34 SFM, a serum-free medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20 ng/ml recombinant rat glial cell line-derived neurotrophic factor, 10 ng/ml recombinant human basic fibroblast growth factor (both from PeproTech, Rocky Hill, NJ, USA), 10 ng/ml mouse epidermal growth factor (Prospec-Tany TechnoGene, Ltd., East Brunswick, NJ, USA), 1,000 U/ml recombinant mouse leukemia inhibitory factor (LIF; Millipore, Billerica, MA, USA), 20 ng/ml platelet-derived growth factor-BB (PeproTech), 1 mmol/l glutamine, 1X insulin-transferrin-selenium (ITS), and 1X B27 supplements (all from Gibco, Grand Island, NY, USA). The mSSCs (f) (5×105 /ml) cultured in a 25 cm^2 flask under these conditions were passaged every 7 days and the culture medium was changed every 2 days. After culturing for 4 weeks, the mSSCs (f) were capable of stably proliferating *in vitro* as mSSCs (l). Trypsin-EDTA (0.25% Invitrogen) and Accutase (1 mg/ml, Sigma) were used to split mSSCs clusters away from MEF feeder cells. To maintain the adherent state of MEF feeder cells, the process of digestion was controlled within no more than 1 min, observed under a light microscope and stopped using the completed culture medium. The mSSC clusters were transferred to a centrifuge tube and centrifuged under 69 x g at 4°C, 3 min after washing with phosphate-buffered saline (PBS).

The mESC (R1) cell line was kindly donated by Dr Shaorong Gao at the School of Life Sciences and Technology at Tongji University (Shanghai, China). The *in vitro* culture and characterization of mESCs (R1) and the induced differentiation of mSSCs into round spermatids (RSs) were performed as previously described (11,12). Briefly, the mESC (R1) cell line was cultured in DMEM (Gibco) supplemented with 1 mmol/l glutamine (Gibco), 100X nucleotide (Millipore), 55 μM β-ME (Gibco), 15% fetal bovine serum (FBS; Gibco) and 1,000 U/ml LIF (Millipore), on the MEF feeder cells. For the induction of sperm differentiation, the mSSCs were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco), 500 ng/ml follicle-stimulating hormone (Sigma), 5 μM vitamin A (Sigma), 0.1 mM testosterone (Sigma), 100X ITS (Gibco), 1 mmol/l glutamine (Gibco), 100X sodium pyruvate (Gibco), and 100X nonessential amino acid (NEAA; Gibco) on mouse testicular fibroblast feeder cells.

AP staining of mSSCs. The mSSC clusters were fixed in 4% paraformaldehyde at room temperature for 20 min and then washed three times with PBS for 15 min. The detector reagents from the AP detection kit (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) were then added and the samples were incubated at room temperature (in the dark) for 15 min. The reaction was terminated by performing three PBS washes. Images were captured using a light microscope (IX71 model with TH4-200 accessories; Olympus, Tokyo, Japan).

Immunohistochemical analysis. The mouse testes were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and processed for immunohistochemical analysis. Briefly, 5-μm section slides were dewaxed in xylene and rehydrated using a series of graded alcohols. Immunostaining was performed by incubating the slides with the mouse monoclonal anti-promyelocytic leukaemia zinc finger (PLZF) antibody (sc-28319; 1:100) overnight at 4°C, followed by incubation with goat anti-mouse IgG-HRP (sc-2005; 1:200) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 37°C for 1 h. The sections of the mouse testes were counterstained with hematoxylin after diaminobenzidine staining (both from Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China) and examined under a light microscope (Olympus).

Immunofluorescence. The mSSC clusters were fixed with 4% paraformaldehyde for 30 min, washed three times with

PBS, and blocked in 1% BSA (Sigma) for 30 min. The cells were incubated with a mouse monoclonal anti-GFRα1 antibody (sc-271546; 1:200; Santa Cruz Biotechnology, Inc.) and an anti-PLZF mouse IgG antibody (sc-28319; 1:200; Santa Cruz Biotechnology, Inc.) at 4˚C overnight and washed three times in PBS. The secondary antibody, Alexa Fluor 568-labeled goat anti-mouse IgG (1:100; Invitrogen) was added and incubated for 1 h at 37˚C in the dark. The cell nuclei were stained with 10 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR, USA). The samples were observed under a fluorescent microscope (IX71 with U-RFL-T accessories; Olympus).

Flow cytometric analysis. The mSSC clusters were digested with Accutase (Stem Cell Technologies, Inc., Vancouver, BC, Canada) and the collected cells were fixed in 4% paraformaldehyde for 20 min followed by three washes with PBS. The cells were then stained with mouse monoclonal anti-CD90.2-FITC (Miltenyi Biotec) for 30 min at 4˚C in the dark and detected by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

RNA extraction, cDNA synthesis, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from mSSCs (f), mSSCs (l), and mESCs using an RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. RNA was transcribed to cDNA using a cDNA synthesis kit (Takara, Otsu, Japan) with oligo-dT primers. The primer sequences used in this study are listed in Tables I and II. Relative mRNA expression analyses were run in triplicate for each sample using a Power SYBR-Green Realtime PCR kit (Toyobo Co., Ltd., Osaka, Japan) on a qPCR machine (Illumina, Inc., San Diego, CA, USA). β-actin was used as an internal control. The relative mRNA abundance of target genes was expressed as $2^{-\Delta\Delta Ct}$.

Western blot analysis. Proteins were extracted from mSSCs (l) and mESCs using RIPA lysis buffer (Beyotime, Shanghai, China) containing 1% protease inhibitor cocktail (Roche, Mannheim, Germany). The lysed samples were centrifuged at 4˚C, 10,000 x g for 15 min to obtain the supernatants. Protein concentrations in the supernatants were determined using the BCA protein assay kit (Bio-Rad, Hercules, CA, USA). The supernatant proteins were denatured, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% non-fat dry milk powder in 1X PBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The blots were incubated with primary antibodies [rabbit anti-mouse PRMT5 (ab2538; 1:200; MultiSciences Biotech Co., Ltd., Hangzhou, China); rabbit anti-mouse LIN28 homolog A (LIN28) (sc-67266; 1:200); rabbit anti-mouse β-actin (sc-130656; 1:1,000) in TBST with 5% non-fat milk overnight at 4˚C with gentle shaking, followed by incubation with peroxidase-conjugated secondary antibody (goat anti-rabbit IgG-HRP; sc-2030; 1:1000) (all from Santa Cruz Biotechnology, Inc.) in TBST with 5% non-fat milk for 2 h at room temperature. Chemiluminescence signals were detected using SuperSignal West Dura HRP detection kits (Pierce, Rockford, IL, USA). The images were captured using a ChemiDoc XRS system equipped with Quantity One software (Bio-Rad).

DNA methylation analysis. Genomic DNA was extracted from mSSCs (l) and mESCs using a Genomic DNA kit (Tiangen Biotechnology, Beijing, China) and treated with an EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) to deaminate unmethylated cytosines to uracils. The DNA templates were used to amplify differentially methylated regions (DMRs) by specific primers (forward, 5'-TGGTTGTTT TGTAGGATTTGTTAGA-3' and reverse, 5'-AAAAC TTCCCTCTTCCCTCTTAATAT-3'). The amplified products were then purified using a Gel Extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA), subcloned into pMD™18-T vectors (Takara) and sequenced by M13R primers.

Statistical analysis. The differences between groups were assessed using ANOVA and Student's t-tests with SPSS v.11 software. The results are presented as the means ± standard error. A $p<0.05$ was considered to indicate a statistically significant difference.

Results

Isolation of mSSCs (f). Immunohistochemical staining of sections of 6-day-old male ICR mouse testes showed that the PLZF-positive mSSCs were localized to the basal membrane of the testicular seminiferous tubules (Fig. 1A). The mSSCs (f), enriched by CD90.2 microbeads, displayed a unified morphological appearance (Fig. 1B) and AP staining activity (Fig. 1C). These mSSCs (f) had a purity of 79.5%, as detected by flow cytometry (Fig. 1D), and immunofluorescence staining confirmed that they expressed the SSC marker, PLZF protein (Fig. 1E-G).

Propagation and characterization of mSSCs (l). The self-renewal capacity of mSSCs (f) was maintained *in vitro* for >5 months [to produce mSSCs (l)] on MEF feeder cells (Fig. 2A and B). The mSSCs (l) displayed AP activity (Fig. 2C) and expressed CD90.2 (Fig. 2D) and GFRα1 (Fig. 2E-G). These colonies of mSSCs (l) were quite different from the colonies of mESCs (Fig. 2H and I). Furthermore, RT-PCR revealed that the mSSCs (l) expressed germline factors (*Plzf*, *Vasa*, *Dazl*, *Nanos3* and *Stra8*), ESC pluripotency factors (*Oct4*, *Sox2*, *Nanog*, *Lin28*, *N-Myc*, *Klf4* and *Tert*) and *Cldn6* and *Pdgfrα* surface markers, whereas MEFs only expressed *N-Myc* and *Klf4* (Fig. 2J).

Differentiation of mSSCs (l). Our results indicated that mSSCs (l) were capable of differentiating into sperm *in vitro*. After 7 days of differentiation culture, A-paired (Apr) spermatogonia were observed (Fig. 3A). Subsequently, A-aligned (Aal) spermatogonia of 4- (Aal-4) (Fig. 3B), 8- (Aal-8) (Fig. 3C) and 16-cells (Aal-16) (Fig. 3D) emerged on days 8, 10 and 11, respectively. Next, A1, A2, A3, A4, intermediate (In), and B spermatogonia began to appear from days 12 to 14 (Fig. 3E and F). During this pivotal developmental time frame, differentiated spermatogonia (A2 to B) derived from A1 cells were synthesized in bulk in preparation for meiosis. Round spermatids (RSs) were formed on day 16 (Fig. 3G) after meiosis. These RSs expressed sperm markers (*Gsg2* and *Acrosin*), whereas mESCs did not express either gene (Fig. 3H).

Table I. Primer sequence, target product size and accession number of target genes for regular PCR.

Gene	Primer sequence (5'→3')	Product size (bp)	Accession no.
β-actin	F: TGCTGTCCCTGTATGCCTCTG R: TGATGTCACGCACGATTTCC	222	NM_007393.3
Oct4	F: GGGATGGCATACTGTGGACC R: CAGAGCAGTGACGGGAACAGA	837	NM_013633.3
Sox2	F: AAACCACCAATCCCATCCAA R: TTGCCTTAAACAAGACCACGAA	459	U31967.1
Nanog	F: CTGATTCTTCTACCAGTCCCAAAC R: AGATGCGTTCACCAGATAGCC	380	XM_006506651.1
Lin28	F: CCAAAGGAGACAGGTGCTACAA R: GGCAGGCTTTCCCTGAGAA	167	XM_006539317.1
N-Myc	F: GGTGGGTCGTCGAGTGCTAG R: AGTGGTTACCGCCTTGTTGTTA	393	M36277.1
Klf4	F: ACTAACCGTTGGCGTGAGGA R: TGCTAACACTGATGACCGAAGG	625	BC010301.1
Tert	F: AGCATTTCACCCAGCGTCTC R: TGCTCGATGACAACGGAGTTC	436	XM_006517210.1
Plzf	F: ACCCATACTGGCACGGACAT R: TGTGAACCCTGTAGTGCGTCTC	346	XM_006510258.1
Vasa	F: AGCATTCCCATTGTATTAGCAGG R: CACTTGCCCAACAGCGACA	573	NM_001145885.1
Dazl	F: GTTAGGATGGATGAAACCGAAAT R: CAGATTTAAGCACTGCCCGAC	739	NM_010021.5
Nanos3	F: CGAGTCCCGTGCCATCTATC R: GGGGCTTCCTGCCACTTT	302	NM_194059.2
Stra8	F: AGGCAACCAACCCAGTGATG R: TCCTGTTCCTGAATATGAATCTTTGT	156	XM_006505829.1
Cldn6	F: GGCAACAGCATCGTCGTGG R: GAAGTCCTGGATGATAGAGTGGGC	333	NM_018777.4
Pdgfrα	F: GTTCAAGACCAGCGAGTTTAATGT R: GCCAAAGGTGGGCTCAATC	376	NM_011058.2
Gsg2	F: CTTTAGTGATTGCCTTTCCACG R: GTGGGAATGGTGCTCGTTTT	612	D87326.1
Acrosin	F: TCTTGGCAGTGTCCGTGGTT R: TGTTTCTTCCATATTCGATTTCTTGT	309	D00754.1

F, forward primer; R, reverse primer.

Relative mRNA expression of transcription factors in mSSCs. The relative mRNA expression of transcription factors (*Oct4*, *Sox2*, *Nanog*, *N-Myc*, *Klf4*, *Esrrb*, *Utf1*, *Dppa2*, *Tbx3*, *Nr5a2*, *Prdm14* and *Klf2*) in both types of mSSC was significantly lower than those in the mESCs (Fig. 4A). For example, the expression of *Oct4*, *Sox2* and *Nanog* in the mESCs was significantly higher than in the mSSCs (l). Notably, the expression level of *Prmt5* and *Lin28* was significantly higher in the mSSCs (l) versus the mESCs. Western blot analysis also confirmed that the mSSCs (l) and the mESCs expressed LIN28 and PRMT5 proteins (Fig. 4C). The mRNA expression of *Dmrt1* in both the mSSC types was higher compared with that in the mESCs (Fig. 4A). Additionally, our results indicated that the expression of *N-Myc*, *Dppa2*, *Tbx3*, *Nr5a2* and *Prmt5* in the mSSCs (l) was markedly upregulated in comparison with the mSSCs (f) (Fig. 4A). Confirmation of the qPCR products of the transcription factors was also demonstrated (Fig. 4B).

Relative mRNA expression of epigenetic factors in mSSCs. Epigenetic factors critical for promoting pluripotency and

Table II. Primer sequence, target product size and accession number of target genes for RT-qPCR.

Gene	Primer sequence (5'→3')	Product size (bp)	Accession no.
β-actin	F: TGCTGTCCCTGTATGCCTCTG R: TGATGTCACGCACGATTTCC	222	NM_007393.3
Oct4	F: GTGTTCAGCCAGACCACCATC R: CATTGTTGTCGGCTTCCTCC	112	NM_013633.3
Sox2	F: CAAGGAAGGAGTTTATTCGGATTT R: ATCAACCTGCATGGGCATTT	178	U31967.1
Nanog	F: CTGATTCTTCTACCAGTCCCAAAC R: GCTTCTGAAACCTGTCCTTGAGT	156	XM_006506651.1
Lin28	F: CCAAAGGAGACAGGTGCTACAA R: GGCAGGCTTTCCCTGAGAA	167	XM_006539317.1
N-Myc	F: TCCTCTAACAACAAGGCGGTAA R: TGTGCTGCTGATGGATGGG	130	M36277.1
Klf4	F: ACTAACCGTTGGCGTGAGGA R: CGTTGAACTCCTCGGTCTCC	175	BC010301.1
Esrrb	F: CATGAAATGCCTCAAAGTGGG R: TCCTGCTCAACCCCTAGTAGATT	186	NM_011934.4
Utf1	F: TCCTCTTACGAGCACCGACAC R: GAGCAACCTGCGGGGAA	146	NM_009482.2
Dppa2	F: GAGGAGCCAAACACAGACTACG R: CGGAGGACAGGTGCTTGGT	138	AF490346
Tbx3	F: GGAACCCGAAGAAGACGTAGAA R: CTTTTTATCCAGTCCAGAGCACC	160	NM_011535.3
Nr5a2	F: TCCCACACCTGATACTGGAACTT R: GCTTTTCTTGCCTGTTTCGG	114	NM_030676.3
Prdm14	F: GAGTGAGATTTGGACCCTTTCG R: ACCGAGCACAGTTGACATAGGAC	165	NM_001081209
Klf2	F: CCCAGGAAAGAAGACAGGAGTCT R: ACTCAAAGGCATTTCTCACAAGG	122	NM_008452.2
Prmt5	F: CCTTTGCCGACAACGAGC R: AAACTGTGCCTCAGGATCGC	179	NM_013768.3
Dmrt1	F: GGAGCGACAGCGGGTGA R: CGGGTTGCTGGCATTATTCT	142	AF202778.1
Tet1	F: CCTATCTTCCTTCCTAAGCCTCC R: TCAGGGTTTGGTGGGAGTTG	164	NM_001253857.1
Tet2	F: AATGGAAGCCCGTTAGCAGA R: GCACCTGGAATACCCTCTGTCT	150	XM_006501281.1
Tet3	F: GCTCGTCTGGAAGATGCCC R: CTCACGACTCATCTCACGGTTG	120	XM_006505773.1
Parp1	F: CGTCAACTACGAGAAACTCAAAACT R: AGGTCATAGGCGTTGTGCG	120	NM_007415.2
Dnmt1	F: AGTCGGACAGTGACACCCTTTC R: GGTTTCCGTTTAGTGGGGC	118	NM_001199431.1
Kdm2b	F: ACTCACCTTACCGAATTTGAACTG R: ACGTGCTCTTTCAGTACATTCTTTAC	149	NM_001003953.1
Dot1l	F: CTGGCAAGCCTGTCTCCTACTAT R: CGTGGTCGCATTGCTCTTG	149	NM_199322.1

Table II. Continued.

Gene	Primer sequence (5'→3')	Product size (bp)	Accession no.
Max	F: CTCTACACCAACGCCAAGGG R: CAGAAGGAGGATGCGACGAG	178	NM_001146176.1
Tert	F: TGCTGGACACTCAGACTTTGGA R: TTCAACCGCAAGACCGACA	102	XM_006517210.1
Trf1	F: AAGAACGCCTTATCGCAGTTAA R: TCCACTGGTTCTTCGGTTCC	120	NM_009352.3
Zscan4c	F: GCAAATGTTGGTGAAAGCTGTAGT R: TAGTCGGAGCACTCGGGAAG	175	NM_001013765.2

F, forward primer; R, reverse primer.

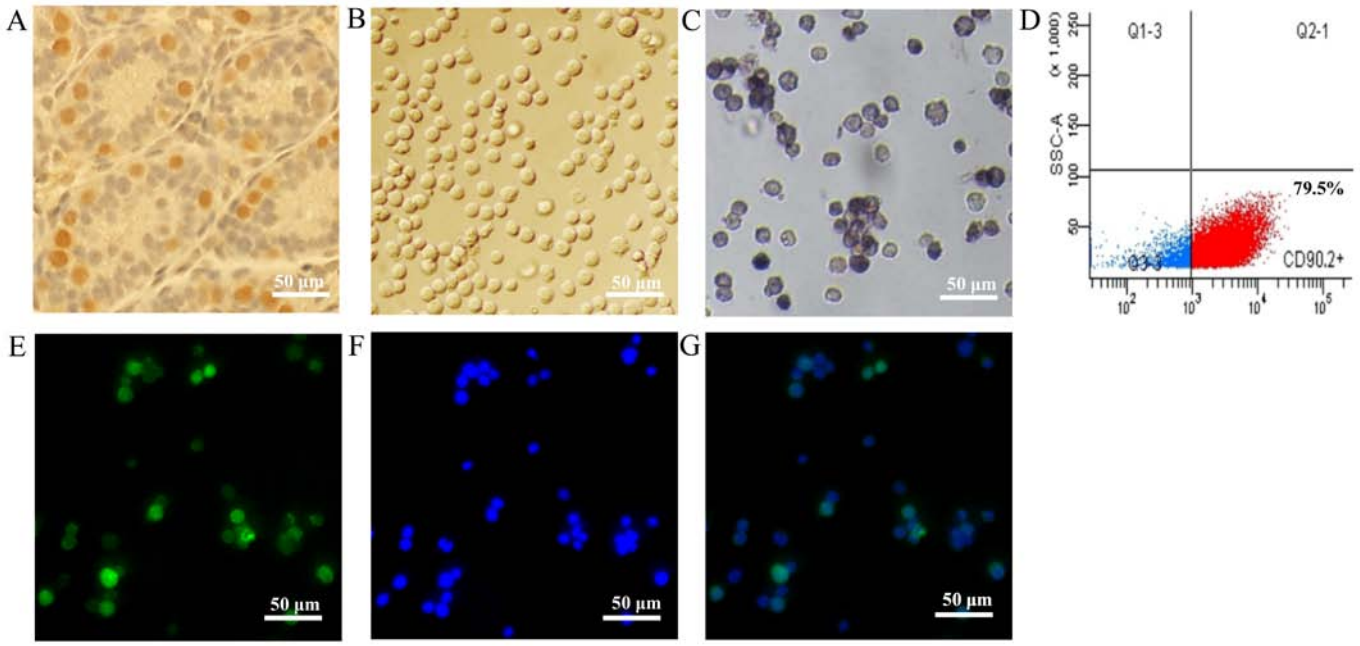

Figure 1. Isolation and identification of mouse spermatogonial stem cells (mSSCs). Representative images of (A) promyelocytic leukaemia zinc finger (PLZF) immunohistochemical staining performed on 6-day-old ICR mouse testes, (B) CD90.2-positive mSSCs enriched by the magnetic-activated cell sorting (MACS) system and (C) freshly isolated mSSCs [mSSCs (f)] exhibiting alkaline phosphatase (AP) staining activity. (D) Flow cytometric analysis of mSSCs (f) performed using the CD90.2 antibody. Representative images of (E) immunofluorescence staining of PLZF in mSSCs (f), and (F) Hochest 33342-stained cell nuclei in mSSCs (f). (G) Merged images of (E and F).

reprogramming were investigated (Fig. 5), including the genes responsible for genomic methylation regulation (*Tet1*, *Tet2*, *Tet3*, *Parp1* and *Dnmt1*, histone modification (*Kdm2b*, *Dot1l* and *Max*), and telomere maintenance (*Tert*, *Trf1* and *Zscan4c*). The results of RT-qPCR revealed that the mSSCs and the mESCs exhibited different expression levels of these factors (Fig. 5A). *Tet1*, *Tet2* and *Zscan4c* were abundantly expressed in the mESCs but not in the mSSCs (l), whereas the levels of *Tet3*, *Parp1*, *Dnmt1*, *Dot1l* and *Tert* were significantly higher in the mSSCs than in the mESCs (Fig. 5A). To further examine the possible association between the low expression of *Tet2* and DNA methylation, we determined the DNA methylation state of the *Tet2* promoter. However, the *Tet2* promoter in the mSSCs (l) did not show a high DNA methylation level by bisulfite sequencing PCR analysis (Fig. 5C). Furthermore, *Kdm2b* expression was significantly higher in the mSSCs (f) than in the mESCs and the mSSCs (l) (Fig. 5A). All three cell types exhibited different expression levels of *Max* (Fig. 5A). Lower levels of *Trf1* were expressed in the mESCs than in the mSSCs (l) (Fig. 5A). Confirmation of the qPCR products of the epigenetic factors was also demonstrated (Fig. 5B).

Discussion

It has been previously demonstrated that the membrane protein CD90.2 was extensively expressed on the surface

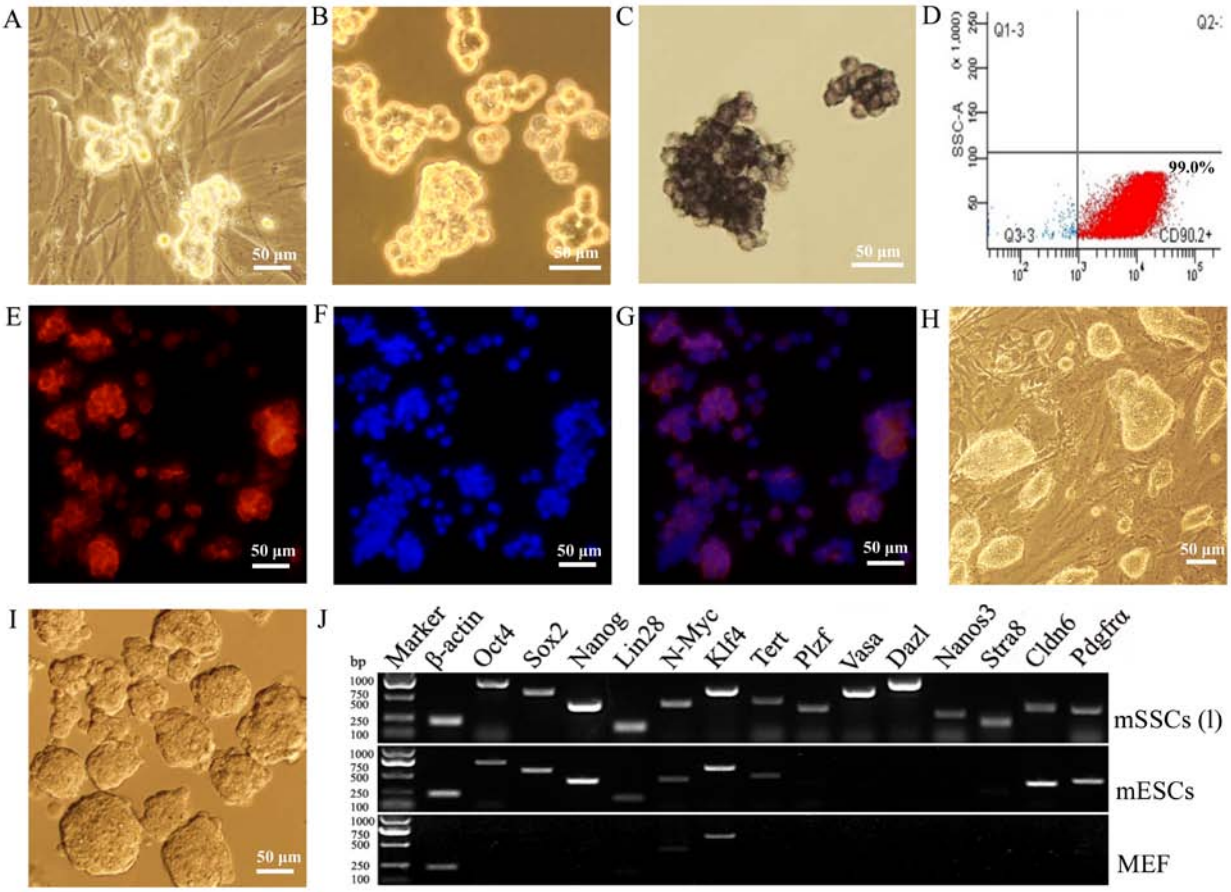

Figure 2. Characterization of long-term propagated mouse spermatogonial stem cells (mSSCs) (l). Representative images of (A) the typical mSSCs (l) colonies on mouse embryonic fibroblast (MEF) feeder cells, (B) the mSSCs (l) colonies without feeder cells, which were used for further analysis, and (C) mSSCs (l) exhibiting alkaline phosphatase (AP staining) activity. (D) Flow cytometric analysis of mSSCs (l) performed using the CD90.2 antibody. Representative images of (E) immunofluorescence staining of GFRα1, (F) Hoechst 33342-stained cell nuclei and (G) merged images of (E and F). Representative images of (H) the colonies of mouse embryonic stem cells (mESCs) cultured on MEF feeder cells and (I) the mESCs colonies without feeder cells, which were used for further analysis. (J) Expression of pluripotency and germ genes was compared in mSSCs (l) and mESCs.

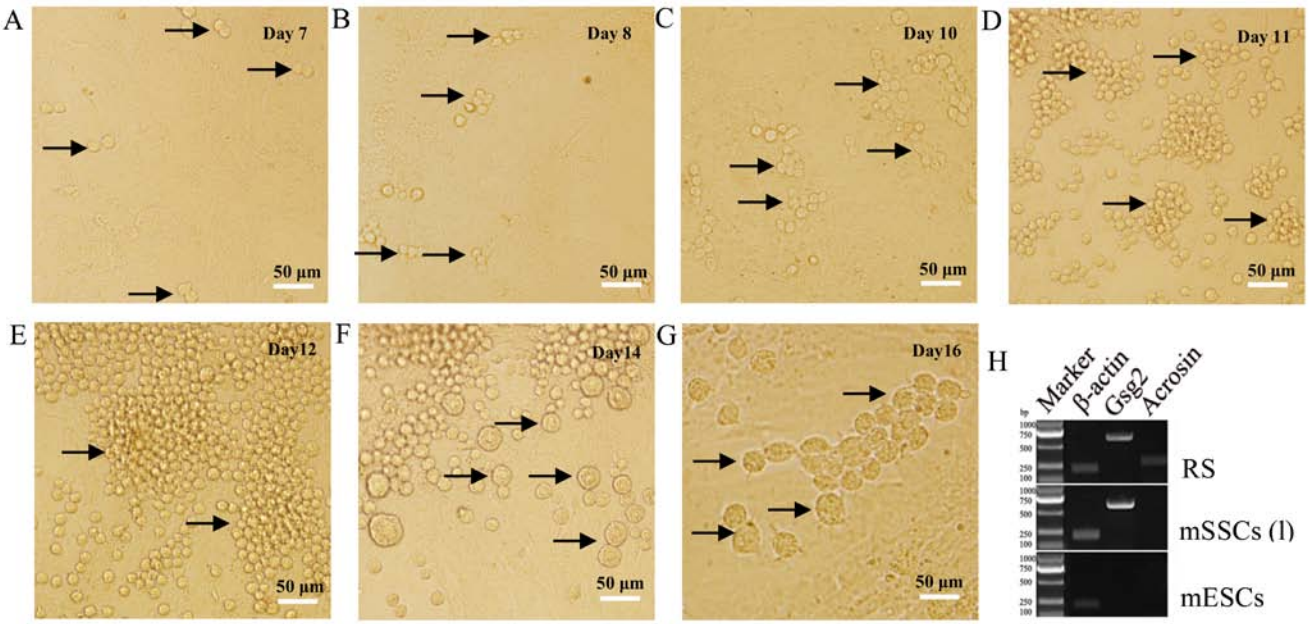

Figure 3. Induction of differentiation of long-term propagated mouse spermatogonial stem cells [mSSCs (l)] into sperms. Representative images showing the following (using black arrows): (A) A-paired (Apr) spermatogonia at day 7, A-aligned (Aal) spermatogonia of (B) 4- (Aal-4) at day 8, (C) 8- (Aal-8) at day 10, (D) and 16- (Aal-16) cells at day 11, and (E) differentiated spermatogonia of A1-A4, intermediate (In) and B spermatogonia at day 12. (F) Bulky cells termed B spermatogonia at day 14. (G) Round spermatids (RSs) appeared with multiple tail cells at day 16. (H) RS exhibited mRNA expression of *Gsg2* and *Arosin*. mESCs, mouse embryonic stem cells.

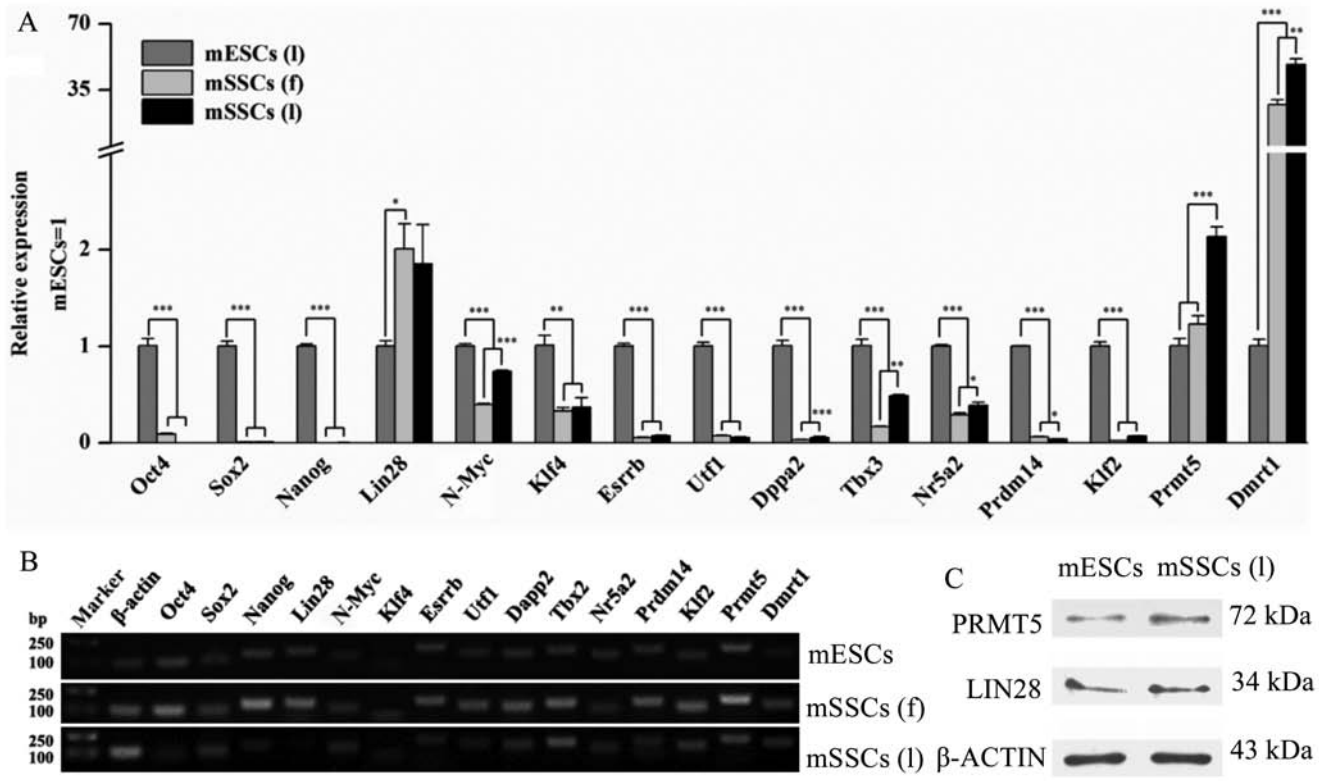

Figure 4. Relative mRNA expression levels of transcription factors in mouse spermatogonial stem cells (mSSCs). (A) Relative mRNA expression of transcription factors in mSSCs is shown, n=3, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$. (B) Confirmation of qPCR products of transcription factors. (C) Western blot analysis of PRMT5 and LIN28 in mouse embryonic stem cells (mESCs) and long-term propagated mouse spermatogonial stem cells [mSSCs (l)]. mSSCs (f), freshly isolated mouse spermatogonial stem cells.

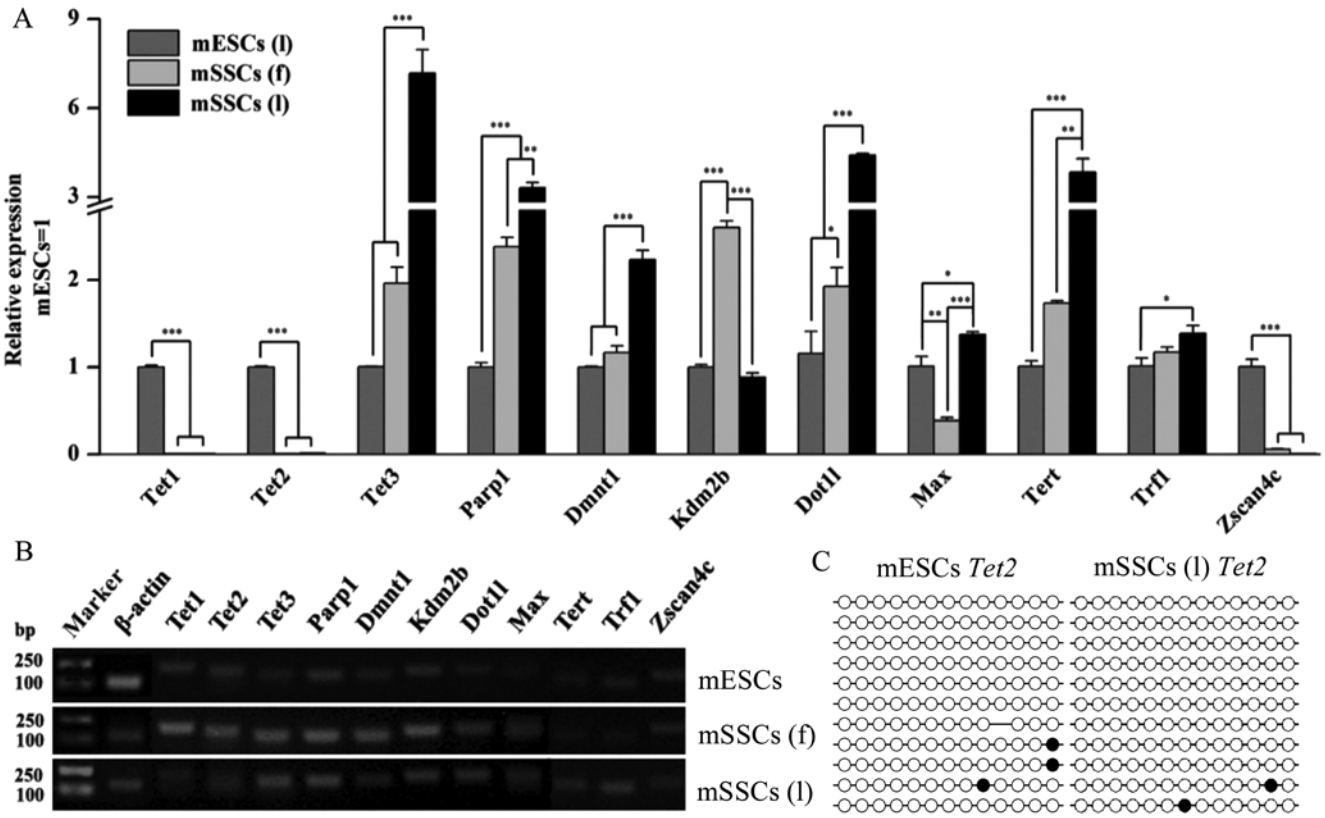

Figure 5. Relative mRNA expression levels of epigenetic factors in mouse spermatogonial stem cells (mSSCs). (A) Relative mRNA expression of epigenetic factors in mSSCs is shown, n=3, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$. (B) Confirmation of qPCR products of epigenetic factors. (C) Analysis of methylation levels of the *Tet2* promoter for mouse embryonic stem cells (mESCs) and long-term propagated mouse spermatogonial stem cells [mSSCs (l)]. mSSCs (f), freshly isolated mouse spermatogonial stem cells.

of mSSCs (13). In addition, the enrichment of mSSCs using CD90.2 microbeads was more efficient than the conventional isolation methods (13). Herein, we observed that the mSSCs (l) exhibited AP activity and expressed the SSC markers, GFRα1 and CD90.2, which is in agreement with previous findings (14). Further experiments demonstrated that the mSSCs (l) expressed germ genes (*Plzf*, *Vasa*, *Dazl*, *Nanos3* and *Stra8*) and pluripotency genes (*Oct4*, *Sox2*, *Nanog*, *Lin28*, *N-Myc*, *Klf4* and *Tert*). *Cldn6* has been identified as a novel surface marker for mouse PSCs (15), and *Pdgfrα* was found to be involved in the regulation of cell division and migration (16). Our results showed that *Cldn6* and *Pdgfrα* were expressed on the mSSCs (l). The successful establishment of mSSCs is characterized by their self-renewal potential and ability to differentiate into sperm (17). Herein, we showed that the mSSCs (l) were capable of differentiating into sperm, by observing the morphological characteristics of mSSCs (l) as well as by determining the expression of the sperm markers, *Gsg2* and *Acrosin*. Collectively, our results suggested that the mSSCs (f) isolated from 6-day-old ICR mouse testes using CD90.2 microbeads may be cultured long-term and maintain the ability to differentiate into sperm.

On the one hand, pluripotency transcriptional networks have been found to be crucial for controlling ESC pluripotency and for somatic cell reprogramming (5,18). Well-known transcription factors, *Oct4*, *Sox2*, *Nanog*, *Lin28*, *N-Myc* and *Klf4,* have been used to induce pluripotency (6,19). However, recent evidence has suggested that the downstream factors, *Esrrb*, *Utf1*, *Lin28* and *Dppa2,* may also promote iPSC production (20). It has been demonstrated that *Tbx3* is essential for pluripotency regulation by regulating the expression of *Tet2*, *Dnmt3b* and *Zscan4* (21). Furthermore, high expression of *Nr5a2* [also known as liver receptor homolog-1 (*Lrh1*)] had the capacity to replace *Oct4* to facilitate reprogramming (22,23). In addition, the germline factors (*Prdm14*, *Klf2* and *Prmt5*) were necessary for primordial germ cell (PGC) specialization and they simultaneously shared the ability to reprogramme PGCs and somatic cells into PSCs (24,25). Our results indicated that the mSSCs (f) and the mSSCs (l) exhibited low expression of most transcription factors (*Oct4*, *Sox2*, *Nanog*, *N-Myc*, *Klf4*, *Esrrb*, *Utf1*, *Dppa2*, *Tbx3*, *Nr5a2*, *Prdm14* and *Klf2*) in contrast with the mESCs. However, using RT-qPCR and western blot analysis, we found a very high expression of *Prmt5* and *Lin28* in the mSSCs (l) indicating that they may be critical for supporting mSSC reprogramming *in vitro*. A previous study has shown that *Lin28*, an abundant protein in ESCs, may repress let-7 microRNA processing, thereby controlling ESC self-renewal and differentiation (26). *Prmt5* may mediate histone methylation and interacted with *Stat3* to stimulate the conversion of the inner cell mass, primordial germ cells, epiblast stem cells, and somatic cells into PSCs (25,27,28). Moreover, it has been demonstrated that the knockdown of *Dmrt1* facilitated mSSC reprogramming (10). Our results also revealed that *Drmt1* was expressed at a high level in both types of mSSCs.

On the other hand, epigenetic mechanisms are important for mammalian development and cellular reprogramming (5). The maintenance of particular gene expression patterns has been attributed to DNA methylation and certain histone modifications (5). Epigenetic factors (*Tet1*, *Tet2*, *Tet3*, *Parp1*, *Dnmt1*, *Kdm2b*, *Dot1l*, *Max*, *Tert*, *Trf1* and *Zscan4c*) may alter genomic methylation and chromatin structure, which is directly associated with pluripotency and reprogramming (5).

The genomic methylation enzymes, *Tet1*, *Tet2*, *Tet3*, *Parp1* and *Dnmt1,* are essential regulators of gene expression and reprogramming. Specifically, *Tet2* and *Parp1* were found to be required for early-stage epigenetic modifications during somatic cell reprogramming (29). In addition, a recent study found that *Tet3* played a possible role in germ cell modification of the zygotic paternal genome (30). We have shown that *Tet3* and *Parp1*, genes involved in genomic methylation, were expressed at a higher level in mSSCs (l) compared with the mSSCs (f) and the mESCs; this may be key to mSSC epigenetic reprogramming. Furthermore, it has been demonstrated that *Parp1* was engaged in the modulation of DNA damage repair and gene transcription, and it promoted epigenetic reprogramming during the early stages of iPSC formation (31). *Dnmt1*, which was found to be involved in sustaining genomic DNA methylation and regarded as a barrier to iPSC reprogramming (10), exhibited higher expression in the mSSCs than in the mESCs in this study. Notably, we found a significantly lower level of *Tet2* in the mSSCs (l) versus the mESCs, which may play a key role in SSC reprogramming. However, this low expression was not due to DNA methylation of the *Tet2* promoter according to our bisulfite sequencing PCR analysis.

Histone-associated modified enzymes (*Kdm2b*, *Dot1l* and *Max*) may change the structure of chromatin to influence gene expression. It has been demonstrated that *Kdm2b* plays a role in anti-senescence and pluripotency and may improve iPSC generation (32,33). A recent study found that histone H3 lysine 79 (H3K79) methytransferase, a crucial epigenetic enzyme for transcriptional regulation, served as a barrier to reprogramming and restrained the expression of *Nanog* and *Lin28* (34). Evidence suggests that Max interacts with histone H3K9 methyltransferases and negatively controls germ cell-specific genes in mESCs (35). We found that there were similar expression levels of *Kdm2b* and *Max* in the mSSCs (l) and the mESCs, indicating their potential roles in facilitating SSC reprogramming. However, *Dot1l* was more highly expressed in the mSSCs (l) implying its possible inhibitory effect in SSC reprogramming. In addition, the lower expression of *Max* in the mSSCs (f) versus the mESCs and the mSSCs (l) may contribute to sustained high levels of germline factor expression for gametogenesis.

Telomere maintenance is essential for chromosome stability, cell replicative capacity, and the induction and establishment of pluripotency (36,37). It has been demonstrated that *Tert* (38), *Trf1* (36) and *Zscan4c* (37) were involved in the modulation of telomere length, thus, markedly improving reprogramming efficiency and iPSC quality (39). We observed the high expression of *Tert* and *Trf1* in the mSSCs (l) and *Zscan4c* in the mESCs; this may provide new insights into mSSC reprogramming.

Taken together, our results suggested that the mSSCs exhibited high expression of pluripotency-associated factors (*Lin28* and *Prmt5*), as well as the expression of crucial epigenetic factors (*Tet3*, *Parp1*, *Max*, *Tert* and *Trf1*) that may promote reprogramming. However, the high expression of *Dnmt1*, *Dmrt1* and *Dot1l*, and the low expression of *Tet1* and *Tet2* in mSSCs (l) may be an obstacle for mSSC reprogramming.

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