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, Parpl, Max, Tert and Trf1, in comparison with the mESCs, with the levels of Prmt5, Tet3, Parpl 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, Parpl, 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.

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 Dnmt1, Dnmt3 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 ESCs 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 more than three times and 30 mice were used each time. The mice were sacrificed by decapitation and the testes were removed for further processing. 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 cm² 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 leukemia zinc finger (PLZF) antibody (sc-23819; 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) 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-CBD-90.2-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^ΔΔCt.

**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 membranes were blocked with 5% non-fat dry milk powder in 1X PBS containing 0.1% Tween-20 (TBST) with 5% non-fat milk overnight at 4˚C, 10,000 x g for 15 min to obtain the supernatants. The supernatant proteins were denatured, separated by SDS-PAGE, and transferred to nitrocellulose membranes using the BCA protein assay kit (Bio-Rad, Hercules, CA, USA). 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; 1:1,000) (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'-TGGTTGTTTGTTAGATTGTTA-3' and reverse, 5'-AAACC TTCCCTCTTCCCTTAAAT-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 (l) expressed 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 Pdgfra 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) spermatagonia 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).
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...
| Gene    | Primer sequence (5'→3') | Product size (bp) | Accession no. |
|---------|--------------------------|-------------------|---------------|
| β-actin | F: TGCTGTCCCCCTGTATGCTCTCTG  
          R: TGGATGCACGCAGATTTTCC | 222              | NM_007393.3   |
| Oct4    | F: GTGTTTCAGCCAGACACACCATC  
          R: CATGTGTGCGCTCCCTCCC | 178              | NM_013633.3   |
| Sox2    | F: CAAGGAAGGATTATATTGAGGATTT  
          R: ATCAACCTGCACTGGCATTTT | 156              | U31967.1      |
| Nanog   | F: CTGATTCCTTCTACAGTCCAAACAC  
          R: GCCTTCTGAAACCTGTCTTGAGT | 167              | XM_006506651.1|
| Lin28   | F: CCAAAGGAGACAGATGGTCTCAA  
          R: GCAGGCTTCTCCTGGAGAA | 178              | XM_006539317.1|
| N-Myc   | F: TCCTCTAAACAAACAGGCGGTAA  
          R: TGGTCTGCTGATGATGGG | 130              | M36277.1      |
| Klf4    | F: ACTAACCTGCTGGAGGGAGGA  
          R: CGTTGAACTCCCTGCTCC | 175              | BC010301.1    |
| Esrrb   | F: CATGAAATGGGCTTCAAGGTGGG  
          R: TCCTGCTGATACCCCTTATGAGT | 186              | NM_011934.4   |
| Utf1    | F: TCCTCTACAGGACAGCGACAC  
          R: GAGCAACCTGCGGGGA | 146              | NM_009482.2   |
| Dppa2   | F: GAGGAGCCAACACACAGTACCG  
          R: CGGAGGACAGGTGCTGCT | 138              | AF490346      |
| Tbx3    | F: GGAACCCGAGAAGAGCGTGGAA  
          R: CCTTTTATTCGCGTCCAGACA | 160              | NM_011535.3   |
| Nr5a2   | F: TCCACACCTGTACTGGAACCTT  
          R: GCCTTCTGCTGCTGCTGTT | 114              | NM_030676.3   |
| Prdm14  | F: GAGTGAGATTTTGAGGACCTTTG  
          R: ACCGAGCACTTGGACATAGGAC | 165              | NM_001081209  |
| Klf2    | F: CCCAGGAAAGAAGACAGGAGTCT  
          R: ACTCAAAGGCATTCTCACAGG | 122              | NM_008452.2   |
| Prmt5   | F: CTTTTCGCGAACAACAGGC  
          R: AAACGTTGCTCCAAGATGCG | 179              | NM_013768.3   |
| Dmnt1   | F: GGACGACAGCAGGGGTA  
          R: CGGGTGCTGCGCATATTCT | 142              | AF202778.1    |
| Tet1    | F: CCTATCTCTCTCTCTCAAGCTCCC  
          R: TCAGGTTTGGTGGGAAGTGG | 164              | NM_001253857.1|
| Tet2    | F: AATGGGAAGCCTCAGCAGA  
          R: GCATTGCTGCTACCTGCC | 150              | XM_006501281.1|
| Tet3    | F: GCTGCAGTAGAGATGCCC  
          R: CTCACGACTCATCTCAGGTTG | 120              | XM_006505773.1|
| Parp1   | F: CGTACACTACGAGAAACACTCAAACCT  
          R: AGGTCTCTGGTGCTCTGG | 120              | NM_007415.2   |
| Dmnt1   | F: AGTCGAGCTAGCTGACACCT  
          R: GTTCTCTCTGCTGTTGGG | 118              | NM_001199431.1|
| Kdm2b   | F: ACTACACCTACGGAATTTGAACCT  
          R: ACGTGCTCTTCTACATCATTTT  
          R: CGTGGTGCACTTGCTTGTGG | 149              | NM_001003953.1|
| Dot11   | F: CGTGGCAAGCTGCTCCTCCTACTAT  
          R: CGTGGTGCACTTGCTTGTGG | 149              | NM_199322.1   |
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 bisulphite 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 (Pufy, 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 Pdgfra was found to be involved in the regulation of cell division and migration (16). Our results showed that Cldn6 and Pdgfra 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 Tert2, Dmnt3b 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 Dnmt1 facilitated mSSC reprogramming (10). Our results also revealed that Dnmt1 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 Tert2 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 Tert2 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) methyltransferase, 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, Dnmtl and Dot1l, and the low expression of Tert1 and Tert2 in mSSCs (l) may be an obstacle for mSSC reprogramming.
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