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Characterization of Immortalized Dairy Goat male Germline Stem Cells (mGSCs)

Haijing Zhu, Jing Ma, Rui Du, Liming Zheng, Jiang Wu, Wencong Song, Zhiwei Niu, Xin He, Enqi Du, Shanting Zhao, and Jinlian Hua*

College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering & Technology, Key Lab for Animal Biotechnology of Agriculture Ministry of China, Northwest A&F University, Yangling, Shaanxi 712100, China

ABSTRACT

Male germline stem cells (mGSCs), in charge for the fertility in male testis, are the only kind of adult stem cells that transmit genetic information to next generation, with promising prospects in germplasm resources preservation and optimization, and production of transgenic animals. Mouse male germline stem cell lines have been established and are valuable for studying the mechanisms of spermatogenesis. However, there is a lack of stable mGSC cell lines in livestock, which restricts the progress of transgenic research and related biotechnology. Here, we firstly established an immortalized dairy goat mGSC cell line to study the biological properties and the signaling pathways associated with mGSCs self-renewal and differentiation. The ectopic factors SV40 large T antigen and Bmi1 genes were transduced into dairy goat mGSCs, and the results showed that the proliferation of these cells that were named mGSCs-I-SB was improved significantly. They maintained the typical characteristics including the expression of mGSC markers, and the potential to differentiate into all three germ layers, sperm-like cells in vitro. Additionally, mGSCs-I-SB survived and differentiated into three germ layer cell types when they were transplanted into chicken embryos. Importantly, the cells also survived in mouse spermatogenesis deficiency model testis which seemed to be the golden standard to examine mGSCs. Conclusively, our results demonstrate that mGSCs-I-SB present the characteristics of mGSCs and may promote the future study on goat mGSCs. J. Cell. Biochem. 115: 1549–1560, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: MALE GERMLINE STEM CELLS; IMMORTALIZATION; DAIRY GOAT

Male germline stem cells (mGSCs), also named spermatogonial stem cells (SSCs), localized in the basement of seminiferous tubules, are able of self-renewal to maintain the number of mGSCs and differentiation into sperm constitutively which are mainly in charge of supplying the material basis for male fertility. Male GSCs have been studied for many years, especially in mouse and human. Importantly, the immortalized mouse mGSCs have been obtained and the culture system has been developed [Feng et al., 2002; Kubota et al., 2004a,b; Hofmann et al., 2005]. Pluripotent stem cells have been obtained from mGSCs in mouse and human, and are a novel resource for pluripotent cells besides embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [Guan et al., 2006; Conrad et al., 2008]. All these progresses promoted the study of the mechanisms on self-renewal and differentiation of mGSCs and their application in agriculture and life science, such as production of transgenic animals [von Schonfeldt et al., 2004; Kanatsu-Shinohara et al., 2005, 2006; Li et al., 2005; Ballow et al., 2006; Naughton et al., 2006; Shinohara and Kanatasu-Shinohara, 2007; He et al., 2008, 2009; Sikarwar and Reddy, 2008; Izsvák et al., 2010; Kanatsu-Shinohara and Shinohara, 2010; Shi et al., 2010; Niu et al., 2011; Zhang et al., 2011; Suzuki et al., 2012].

As we described previously, dairy goat is important for Chinese people, and studies on dairy goat mGSCs may improve the preservation and optimization of the germplasm resources [Zhu et al., 2013] and provide us a great improvement of quality and quantity of milk and meat. Up to date, there is a lack of stable culture system and mGSC cell line in livestock [Luo et al., 2006; Bi et al.,

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*Correspondence to: Dr Jinlian Hua, College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering & Technology, Key Lab for Animal Biotechnology of Agriculture Ministry of China, Northwest A&F University, Yangling, Shaanxi, China. E-mail: jinlianhua@nwsuaf.edu.cn

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2007; Aponte et al., 2008; Kaul et al., 2010, 2012; Hua et al., 2011; Bahadorani et al., 2012; de Barros et al., 2012; Heidari et al., 2012; Li et al., 2012, 2013; Zhu et al., 2013; McMillan et al., 2013]. The dairy goat mGSCs cannot survive in vitro under specific culture conditions for a long period as previously reported by our laboratory [Zhu et al., 2012]. Thus, it is of great significance to obtain the immortalized dairy goat mGSC cell line, which will be critical for the long-term study of dairy goat mGSCs and other aspects of the cells biology, including dedifferentiation mGSCs into pluripotent state. Feng et al. [2002] and Hofmann et al. [2005] have shown that mGSCs could be immortalized by exogenous factors, such as TERT and Simian virus 40 (SV40) T antigen. These cells shared typical SSC characteristics including the morphology, expression of mGSCs markers such as DAZL and GFRa1, the ability of differentiating into haploid sperm cells and rebuilding seminiferous tubules after transplantation into mouse spermatogenesis deficiency testis.

Immortalization methods in mammals include transduction of primary cells with vectors carrying viral genes, such as Simian virus 40 (SV40) T antigen, Epstein–Barr virus (EBV), Adenovirus E1A and E1B, and human Papiloma virus (HPV) E6 and E7. SV40 large T antigen was regarded as an efficient factor for animal cell immortalization [Lübbe et al., 1983; Petit et al., 1983; Ozer, 2000], and it has been demonstrated to be effective for mouse mGSCs immortalization in 2005 [Hofmann et al., 2005]. Bmi1 is regarded as an marker of mouse mGSCs [Zhang et al., 2008] and promotes stem cell proliferation [Pietersen et al., 2008]. Being an immortalization factor, Bmi1 has been demonstrated to be effective in immortalizing somatic cells [Dimri et al., 2002; Saito et al., 2005]. Conclusively, we believe SV40 large T antigen and Bmi1 are capable of immortalizing dairy goat mGSCs. Here, the lentivirus particles containing SV40 large T antigen and Bmi1 were transduced into primary dairy goat mGSCs, and the cells are immortalized and have been cultured for more than 3 months in vitro. The characteristics of the immortalized mGSCs were determined through the proliferation test, marker detection, the differentiation potential assay in vitro and in vivo. These results suggested that the mGSCs were immortalized by SV40 large T antigen and Bmi1, and these cells will provide unlimited cell resources for studying male germ cell specification and development.

MATERIALS AND METHODS

ISOLATION AND ENRICHMENT OF MALE GERMLINE STEM CELLS (mGSCs) FROM GOAT TESTIS

The adult male dairy goats were killed and their testes were collected. All the procedures were carried on under the supervision of Chinese Association for Laboratory Animal Science, and approved by Shaanxi Centre of Stem Cells Engineering & Technology, Northwest A&F University. Dairy goat mGSCs were isolated and purified as previously reported [Zhu et al., 2012].

PURIFICATION OF DAIRY GOAT mGSCs

The dairy goat testicular cells were cultured on plates treated with 1 μg/ml Fibronectin (Sigma, USA) at 37°C, 5% CO_2 and saturated humidity for 2 h at the density of 1×10^6 cells/ml [Zhu et al., 2012]. Then the attached cells were dissociated with 0.25% Trypsin (Invitrogen, USA) for 5 min at 37°C. The cells were collected and cultured with DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 0.1 mM β-mercaptoethanol (Sigma) and 2 mM glutamine (Invitrogen).

LENTIVIRUS PREPARATION AND THE IMMORTALIZATION OF DAIRY GOAT GERMLINE STEM CELLS

Lentivirus production was referred as Anokye-Danso et al. described [Anokye-Danso et al., 2011]. Briefly, HEK293T cells were seeded on plates 24 h before transfection, then the plasmids containing SV40 large T antigen and Bmi1 along with the plasmids containing pVSVG and pPAX2 were incubated respectively. The virus-containing supernatant was collected at 48 h after transfection, filtered to remove cell debris, and used for transduction. For cell immortalization, dairy goat mGSCs were plated at a density of 1×10^5 cells in a 35 mm dish. Twelve hours later, the cells were transduced with virus-containing supernatant and 10 μg/ml polybrene (Sigma) and incubated overnight at 37°C and 5% CO_2. After 24 h, the medium was discarded and replaced with fresh DMEM/F12 medium (Invitrogen) supplemented with 10% FBS (Hyclone, USA), 0.1 mM β-mercaptoethanol (Sigma) and 2 mM glutamine (Invitrogen), then cultured for more than 1 month until the cells were immortalized. The immortalized cells were named mGSCs-I-SB.

CELL CYCLE ANALYSIS

For cell cycle analysis, dairy goat mGSCs (5th passage) and mGSCs-I-SB (10th passage) were cultured for 48 h and then suspended the cells into single cells and fixed them in 70% ice-cold ethanol for 30 min. After that, cells were incubated with propidium iodide (PI, Sigma) solution supplemented RNase H (Beyotime, China) for 20 min. Cell cycle analysis was determined by flow cytometry as previously reported [He et al., 2008].

POPULATION DOUBLING TIME (PDT) DETERMINATION

The population doubling time (PDT) of dairy goat mGSCs were estimated according to the protocol described previously [Zhang et al., 2011]. Briefly, cells were serially subcultured, the initial seeding cell number and the total cell number cultured 48 h later were all counted respectively. PDT was calculated according to the formula, $PDT = [\log_2/(\log Nt0 - \log N0)] \times t$, where Nt is the number of cells after t h of culturing, N0 is the number of cells seeded.

5-BROMO-2-DEOXYURIDINE (BrdU) INCORPORATION ASSAY

Dairy goat mGSCs proliferative ability was assessed by BrdU Incorporation. Dairy goat mGSCs (5th passage) and mGSCs-I-SB (10th passage) were treated with BrdU (30 μg/ml, Sigma) for 3 h and then subjected to BrdU immunostaining. Cells were fixed with methanol/acetone (V/V = 1:1) for 15 min at room temperature and washed with PBS for three times, incubated with PBS containing 0.1% Triton-100 for 5 min. Then the cells were washed three times with PBS at room temperature. Mouse anti-BrdU (1:100; Santa Cruz) dissolved in 0.1 M PBS (pH 7.4) containing 4% normal goat serum was added and the cells were incubated overnight at 4°C. Cells were washed in PBS for three times, and then incubated with the goat anti-mouse FITC conjugated secondary antibody (1:500, Chemicon) for 1 h at room temperature. After three washes, cells were visualized under fluorescent microscope and analyzed for BrdU staining.

RT-PCR

Total RNA for RT-PCR analysis was extracted from 6th passage dairy goat mGSCs and 11th passage mGSCs-I-SB cultured under normal conditions using RNAiso (TaKaRa, Biotech. Co. Ltd.). The cDNA was synthesized based on 500 μg RNA with a commercially available kit (TaKaRa, Biotech. Co. Ltd.). The PCR steps included denature at 94°C for 5 min, followed by repeated cycles 30 s at 95°C, 55–58°C for 30 s, 72°C for 30–60 s, 35 cycles, and extend at 72°C for 10 min. The primers were designed based on the sequences of the open reading frame from the NCBI GenBank and synthesized by AuGCT Biotechnology (Beijing). The PCR primers and the length of the amplified products are shown in Table 1. The PCR products were analyzed by electrophoresis in 2% agarose (Invitrogen) gel, stained with ethidium bromide (Invitrogen), and visualized under UV illumination.

IMMUNOFLUORESCENCE STAINING

Dairy goat mGSCs (7th passage) and mGSCs-I-SB (13th passage) cultured under normal conditions were fixed with 4% PFA for 15 min at room temperature, followed by three washes in cold PBS for 5 min each. Washed cultures were treated with blocking solution (PBST + 1% BSA) for a minimum of 30 min and incubated in primary antibodies including Oct-4 (1:500, Chemicon), Sox2 (1:500, Chemicon), CD49f (1:500, Chemicon), TERT (1:200, Santa Cruz), Oct4 (1:500, Abcam), Stra8 (1:500, Abcam), Blimp1 (1:200, Santa Cruz), Nanog (1:200, Chemicon). The appropriate FITC conjugated secondary antibodies were used according to the manufacturer's manual (1:500, Chemicon). The nuclei of cells were stained by Hoechst 33342. At the same time, the negative controls were stained with the appropriate fluorescent-conjugated secondary antibodies [Yu et al., 2014].

IN VITRO DIFFERENTIATION OF mGSCs-I-SB

The 15th passage immortalized cells were dissociated into single cell suspension and resuspended in DMEM (Invitrogen) containing 20% FBS (Hyclone), 2 mM L-glutamine (Invitrogen), 1% non-essential amino acids (Millipore), 100 U/ml penicillin and 100 mg/ml streptomycin at 800–1,000 cells/25 μl to form cell clusters. For further differentiation, the embryoid bodies (EBs) (d3) cultured in Petri dishes coated with 0.1% gelatin (Sigma) and DMEM medium containing 20% FBS (Hyclone), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen) and 0.1 mM non-essential amino acids (Invitrogen) for 3–14 days to investigate the potentiality of spontaneous differentiation [Zhu et al., 2012]. And EBs were plated to induce the cells differentiate into adipocyte, osteoblast and cartilage as previous study [Hua et al., 2011; Hu et al., 2012; Qiu et al., 2012]. After differentiation, the cells were determined by histochemistry and immunofluorescence staining with the antibodies of all three germ layers: NSE (Ectoderm), Islet1 (Mesoderm and Endoderm), PDX1 (Endoderm) [Cao et al., 2011; Hua et al., 2011; Qiu et al., 2012].

DETECTION OF THE DIFFERENTIATION POTENTIAL OF mGSCs-I-SB IN VIVO

The 17th passage of immortalized cells cultured under normal conditions were transduced with the lentivirus expressing GFP for 12 h, then the cells were dissociated, collected and 0.5 μl working solution containing the cells with an concentration of 10^7 cells/ml injected into dorsal neural tube of 2.5 days chicken embryos (E2.5) to evaluate whether the cells can differentiate into all three germ layers in vivo. Then the injection hole was sealed with scotch tape and the egg was returned to incubator until E6 for analysis.

After 3.5 days development, the chicken embryos were fixed with 4% PFA for 24 h at 4°C, the oscillation sliced slides were produced and analyzed through immunofluorescence staining. The spinal cords were coronally section at 80 μm by vibration microtome (VT 1000S, Leica, Germany). The sections were washed three times in 0.1 M PBS for 15 min each and then incubated at 4°C with primary antibodies for 12 h. All three germ layers markers and the germline specific markers were detected by immunofluorescence staining assay [Elena de Bellard and Bronner-Fraser, 2005; Boulland et al., 2010]. Then sections were incubated with secondary antibodies for 3 h at room temperature and then washed four times for 15 min each at room temperature. The sections were then counterstained

TABLE 1. The Primers Were Used in Determining the Immortalized Dairy Goat mGSCs

Gene	Sense primer	Antisense primer	Product size (bps)	T_m (°C)	Reference sequence
β-Actin	GCGGCATCCACGAAACTAC	TGATCTCCTTCTGCATCCTGTC	138	58	NM_001101.3
VASA	GCTGGCGTAATAGCGAAGAGG	GCACAGATGCGTAAGGAGAAAA	107	58	KC189826.1
Gfra1	ATTTTATTACCTGCTGCCA	ATTTCAATCATTCCTTCAT	197	51	NM_010279.2
CD117	TCCCAAACCTCAACACCGACAG	GTGTAAGTGCCTCCTTCAGTCCC	153	58	NM_013598
CD90	GATCCAGGACTGAGCTCTCGG	TCACGGGTCAGACTGAACTCATAC	195	58	NM_006288
CD49f	CGAAGCACGAATCCCGAGAC	TGCTCTACACGAACAATCGCTTT	235	58	NM_008397
Oct4A	GACACCTGGCTTCCGACTTC	GCTGAACACCTTCCCAAAGAG	533	59	NM_001265584.1
Bmi1	CCAGAGGGATGGACTGACGA	GGGAACTGTGGGTGAGGAGA	147	59	NM_007552.4
Stra8	AAGGACAGCGGGGTTGAC	TCGGGTTTTTTTGAGTTGC	170	56	JQ836663.1
PLZF	CACCGCAACAGCCAGCACTAT	CAGCGTACAGCAGGTCATCCAG	127	58	JX047313.1
NSE	AAGGACAAATACGGCAAGG	CAGGTCATCACCCACAATC	371	58	JN887466.1
Cyclin D1	TGAACTACCTGGACCGCT	CAGGTTCCACTTGAGYTTGT	212	58	NM_053056.2
Sox2	GGCGGCAACCAGAAGAACAG	GCATCTTGGGGTTCTCCTGG	109	58	JQ290347.1
Nanog	GGAACTGCTGGGGAAAATTA	TACAAATCTTCAGGCTGTATGTTG	118	58	FJ970651.1
CDK2	GCCAGGAGTTACTTCTATGC	TGGAAGAAAGGGTGAGCC	180	58	NM_001798.3
PCNA	AGTGGAGAACTTGGAAATGGAA	GAGACAGTGGAGTGGCTTTTGT	167	58	NM_011045.2
exBmi1	TGTTCCCATAGTAACGCCAATA	GGCATCAATGAAGTACCCTCC	644	58	
SV40	TGACCTCCATAGAAGACACCG	CAAATACCTCAGTTGCATCCC	386	58	

with DAPI (1:1,000, Sigma) for 5 min, washed three times for 5 min each, and finally mounted with a fluorescent mounting medium (Dako, Carpinteria, CA) on glass slides.

TRANSPLANTATION OF THE CELLS INTO RECEIPT SPERMATOGENESIS DEFICIENCY MOUSE TESTIS

Male GSCs-I-SB (20th passage) were dissociated, and the cells were collected through centrifuging at 1,500 r/min for 5 min. The cells were counted and diluted with DMEM/F12 (Invitrogen) supplemented with 10% FBS, and 10^5 cells/testis were then transplanted into seminiferous tubules of the eight receipt spermatogenesis deficiency mouse, which were injected with 40 mg/kg weight busulfan (Sigma) and evaluated by previous study [Choi et al., 2004]. After 2 months, the treated mice were killed, and the recipient's testes were collected. The characteristic of mGSCs-I-SB was analyzed through supervision for GFP expression of the seminiferous tubules and paraffin slides which were produced from the mouse testis transplanted with the immortalized cells and untransplanted [Russell et al., 1996]. This was regarded as the golden standard to determine whether the putative cells maintain the capabilities of mGSCs which were established by Brinster and Avarbock [1994] and Brinster and Zimmermann [1994].

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Statistical comparisons were assessed with analysis of Student's t test. $P < 0.05$ was considered statistically difference and $P < 0.01$ was considered significantly difference.

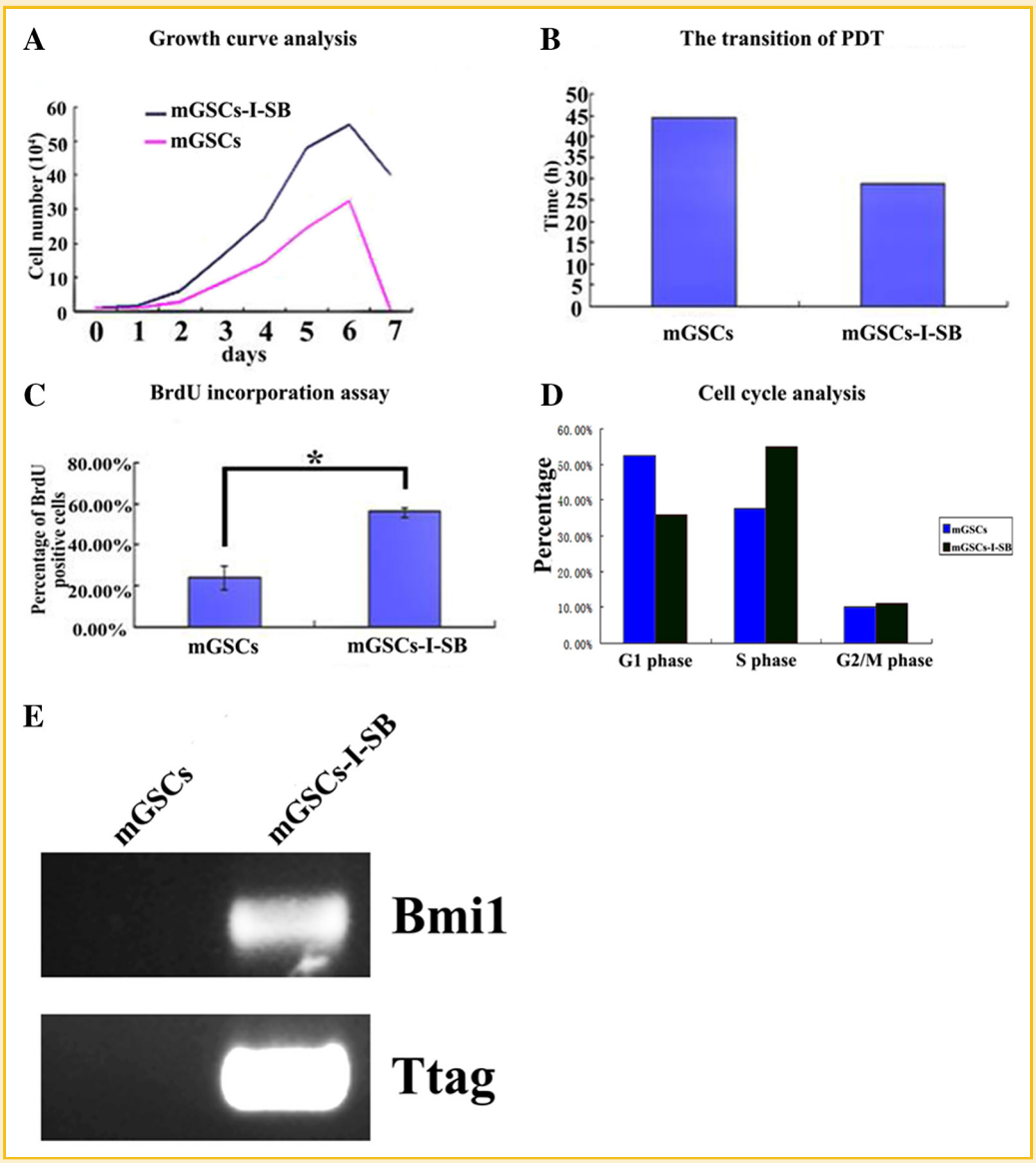

Fig. 1. Dairy goat mGSCs-I-SB maintain greater proliferation ability compared with mGSCs. A: Growth curve. B: PDT analysis. C: BrdU Incorporation assay. D: Cell cycle analysis for mGSCs-I-SB and wild mGSCs. E: PCR detection of the exogenous gene integration.

RESULTS

THE CELLS' PROLIFERATION POTENTIAL BEFORE AND AFTER IMMORTALIZATION

We isolated and enriched dairy goat mGSCs as we described previously [Zhu et al., 2012]. Then the cells were immortalized by the lentivirus carrying SV40 large T antigen and Bmi1 genes, and the mGSCs-I-SB showed an obviously stronger proliferation than wild mGSCs. Growth curve analysis showed that mGSCs-I-SB had a higher cell number compared with dairy goat mGSCs (Fig. 1A). PDT detection showed mGSCs-I-SB hold a lower PDT (28.5 h) to mGSCs (45 h), which means an improvement of proliferation by immortalization (Fig. 1B). BrdU Incorporation assay showed that over 50% of mGSCs-I-SB were BrdU positive compared with 20% positive in

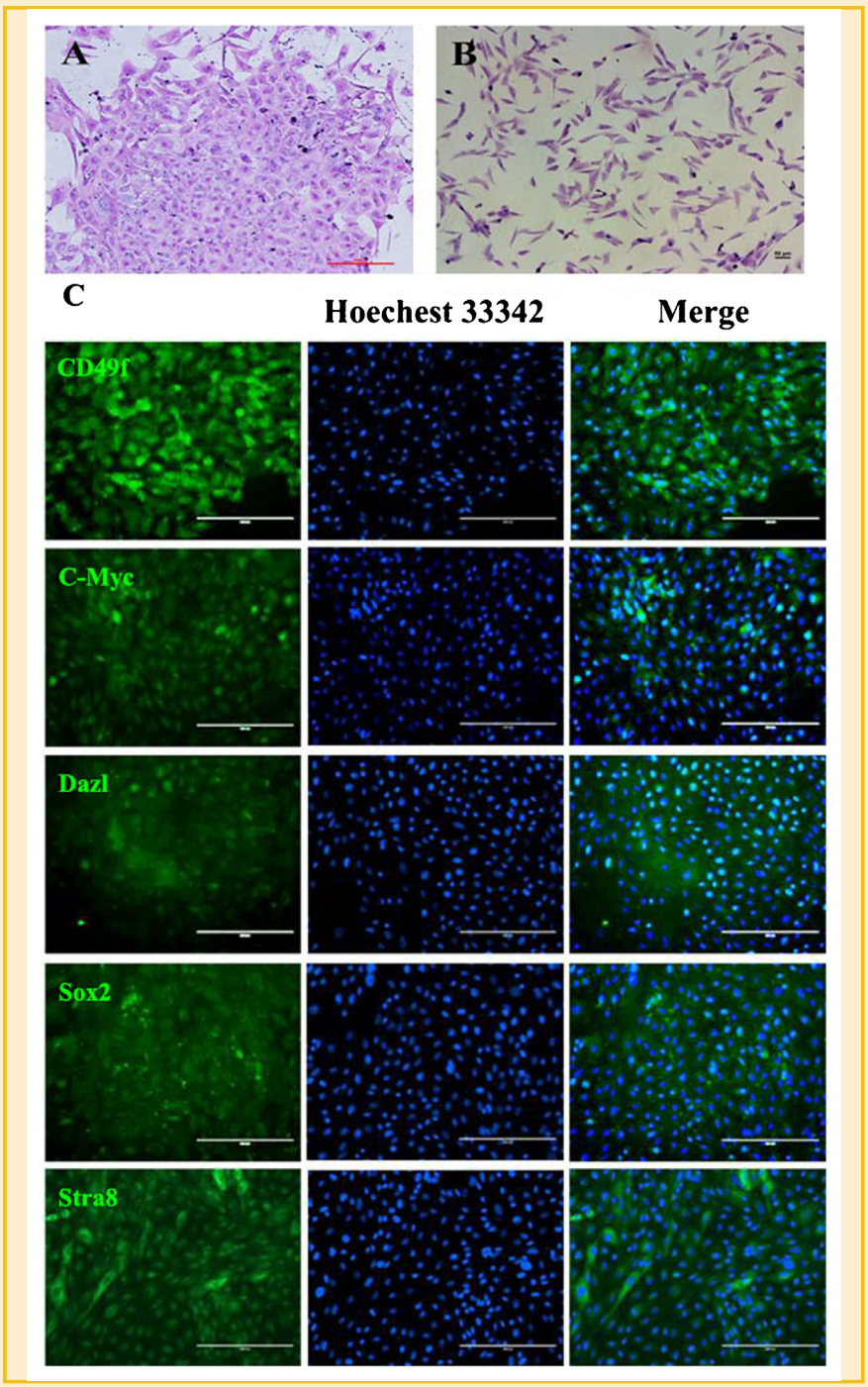

Fig. 2. The morphology alteration of mGSCs-I-SB from wild dairy goat mGSCs. The morphology from wild mGSCs (A) (Bar = 100 μm) to mGSCs-I-SB (B) (Bar = 50 μm) and immunofluorescence staining for wild mGSCs with antibodies including CD49f, c-Myc, DAZL, Sox2, and Stra8 (C) (Bar = 200 μm).

dairy goat mGSCs (Fig. 1C). Also, cell cycle analysis showed that dairy goat mGSCs to mGSCs-I-SB alteration accompanied an increase of S phase cell ratio from 37.7% to 55.0% (Fig. 1D). Moreover, the cells lifespan also showed a great improvement from less than 10 passages to be easily subcultured up to 30 passages. In summary, all these results demonstrated that mGSCs-I-SB maintain a greater potential in proliferation compared to mGSCs.

To evaluate whether the cells were actually immortalized by SV40 large T antigen and Bmi1, we examined the genomic integration of the exogenous genes. As indicated in Figure 1E, the exogenous genes-SV40 large T antigen and Bmi1 were positive in mGSCs-I-SB and negative in mGSCs. This further demonstrated that the immortalization of dairy goat mGSCs was mediated by SV40 large T antigen and Bmi1.

THE MARKERS EXPRESSED IN DAIRY GOAT mGSCs-I-SB

The mGSCs-I-SB exhibited spindle-like cell morphology compared to the epithelioid-like cell morphology before immortalization (Fig. 2A, B). Whether mGSCs-I-SB expressed the canonical mGSC markers seem to be important to supply an intuitive evidence for mGSCs. We detected the mGSC markers through RT-PCR and immunofluorescence staining for the cells before and after immortalization. The immunofluorescence staining showed dairy goat mGSCs were positive for most of the male germ cell markers, such as CD49f, DZAL, Stra8, Sox2, and C-Myc (Fig. 2C) before immortalization, and mGSCs-I-SB were also positive for CD49f, CD90, Blimp1, C-Myc, Nanog, PLZF, Oct4, Stra8, and TERT (Fig. 3A). The RT-PCR results showed that the cells were positive for SSC specific markers such as VASA, GFRa1, CD117, CD90, CD49f, Stra8, PLZF; they were also positive for pluripotency markers including Oct4A, Nanog, Sox2; and proliferative markers such as CDK2, CyclinD1, PCNA, and Bmi1 (Fig. 3B).

THE DIFFERENTIATION POTENTIAL OF mGSCs-I-SB IN VITRO

The cells' pluripotency still need to be elucidated although the associated markers were expressed. Then we examined the differentiation potential in vitro by inducing the cells differentiation into osteoblasts, chondrocytes, neural-like cells and adipocytes. The EBs formed in suspension were collected and cultured under defined solutions. When the cells were induced for a defined protocol as we described previously [Hua et al., 2011], the cells displayed positive for Oil Red O (adipocyte), Alcian blue (chondrocyte), Alizarin Red (osteoblast) (Fig. 4A), and immunocytochemical staining showed

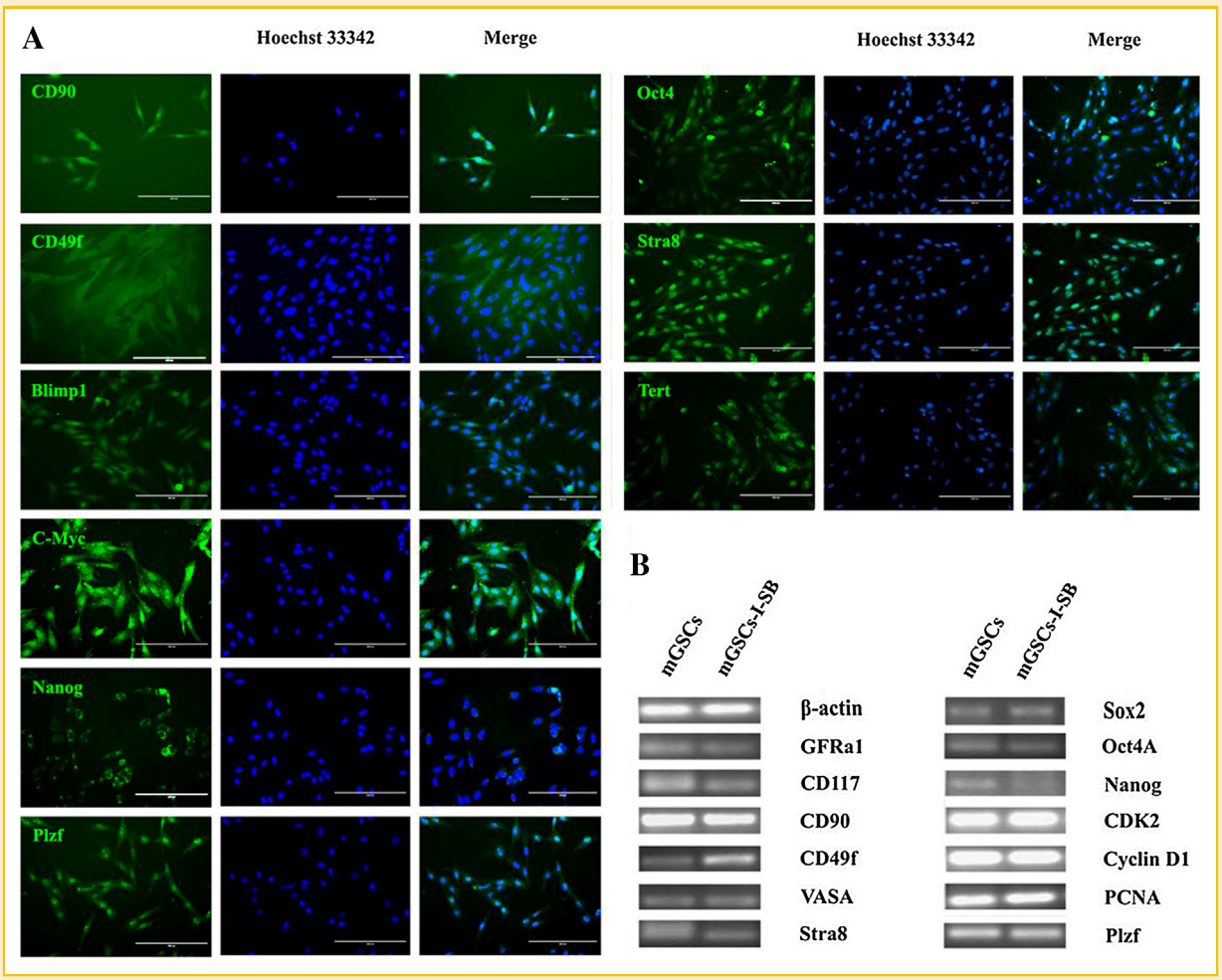

Fig. 3. Detection of the markers of mGSCs-I-SB. Immunofluorescence staining for mGSCs-I-SB with antibodies including CD90, CD49f, Blimp1, c-Myc, Nanog, PLZF, Oct4, Stra8 and TERT (A) (Bar = 100 μm). RT-PCR detection for markers: Gfra1, CD117, CD90, CD49f, Vasa, Stra8, Sox2, PLZF, Oct4A, Nanog, CDK2, CyclinD1, and PCNA (B).

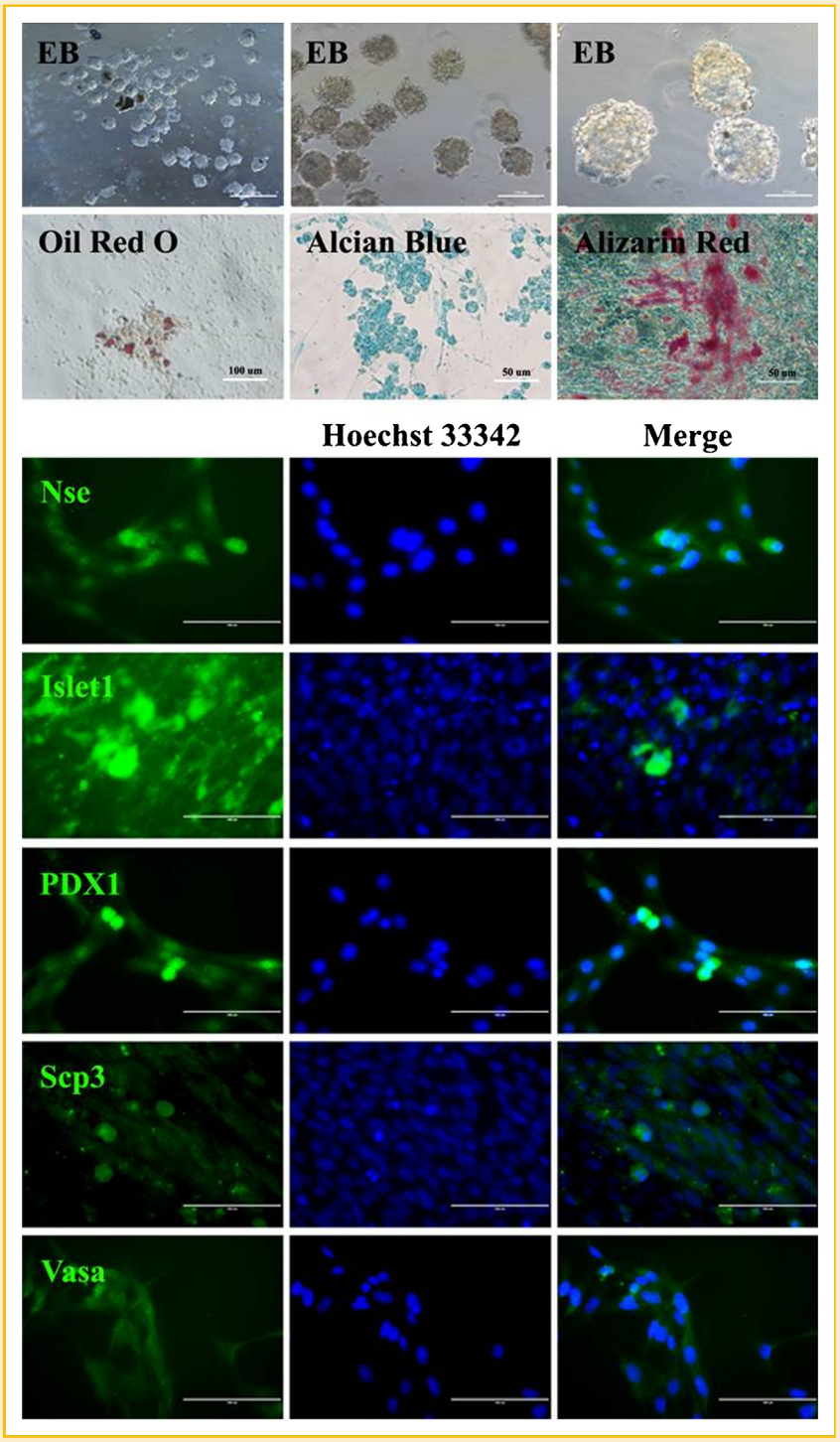

Fig. 4. In vitro differentiation potential determination for mGSCs-I-SB. In vitro, mGSCs-I-SB can form EBs (from left to right, Bar = 400, 200, 100 μm), then differentiate into oil red O (Bar = 100 μm), alcian blue (Bar = 50 μm) and alizarin red (Bar = 50 μm) positive cells in appropriate conditions (A). The cells exhibited positive for NSE, Islet1, PDX1, Scp3, and VASA analyzed by immunofluorescence staining (B) (Bar = 100 μm).

that the cells were differentiated into cells positive for NSE (ectoderm marker), Islet1 (mesoderm and endoderm marker), PDX1 (endoderm marker), Scp3 and VASA (germ cell marker) (Fig. 4B). All these results suggested that mGSCs-I-SB maintain the potential to differentiate into any of the three germ layers in vitro.

THE DIFFERENTIATION POTENTIAL OF mGSCs-I-SB IN VIVO

To determine the differentiation potential of mGSCs-I-SB in vivo, the GFP labeled cells were transplanted into chicken embryos' dorsal neural tube under the protocol illustrated in Supplemented Fig. S1. After 3 days, the GFP positive cells were found in recipient's neural

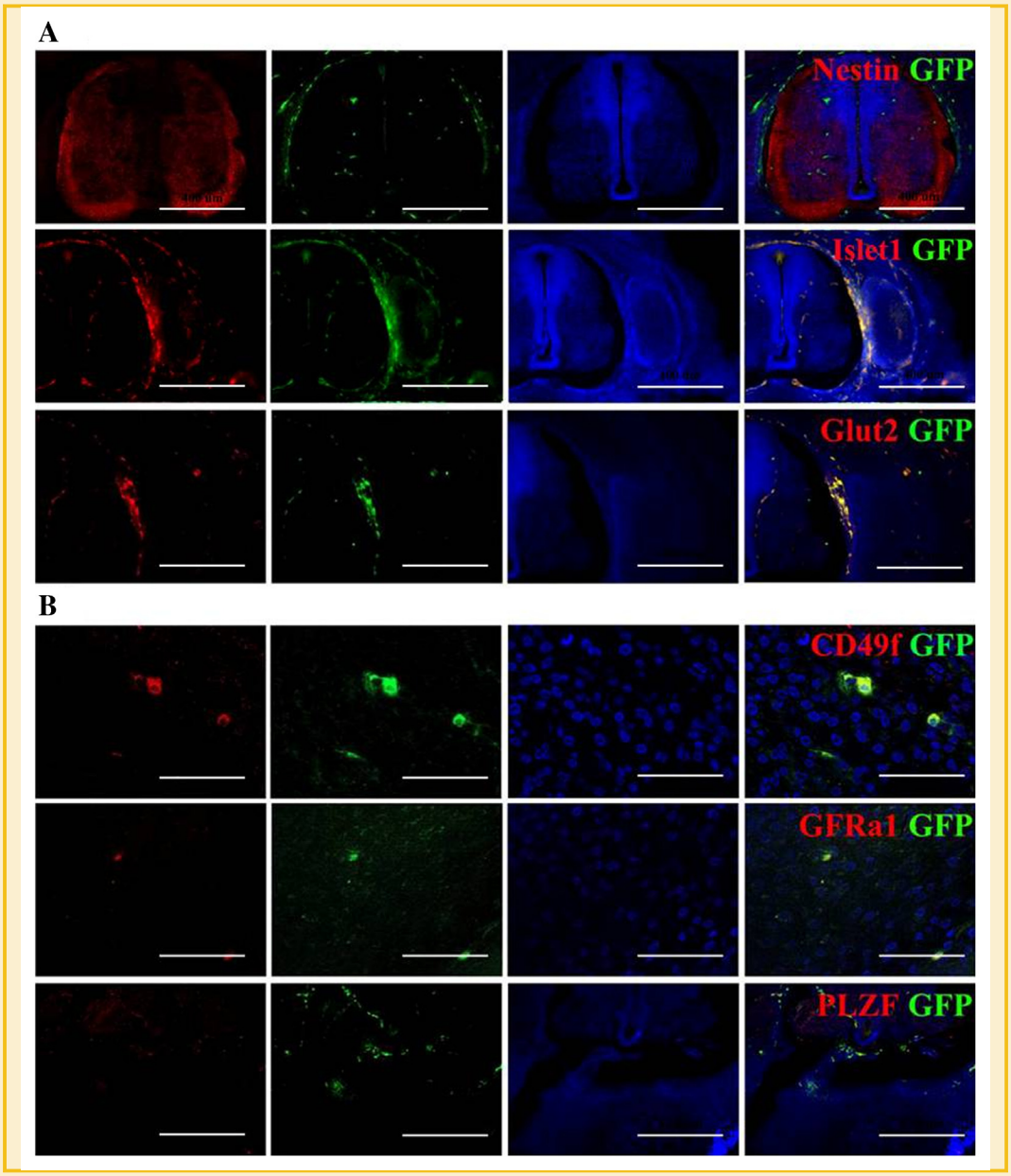

Fig. 5. In vivo differentiation potential of mGSCs-I-SB. When mGSCs-I-SB cells were transplanted and developed in chicken embryo for 3 days, the cells expressed all three germ layers markers including Nestin, Islet1, Glut2 (A) (Bar = 400 μm) and CD49f, GFRa1, PLZF (B) (Bar = 63.5 μm).

tube and out everywhere (Fig. 5). The GFP positive cells were also positive for the markers of all three germ layers including Nestin, Islet1, and Glut2 (Fig. 5A), and the mGSC markers including CD49f, GFRa1, and PLZF (Fig. 5B). All these results evidenced that the cells maintain the differentiation potential to all three germ layers and male germ cell lineages in vivo.

THE CELLS POTENTIAL TO SURVIVE IN MOUSE IMPAIRED SEMINIFEROUS TUBULES

The most important characteristic of mGSCs is the potentiality to reconstitute the impaired seminiferous tubules. The transplantation assay showed that mGSCs-I-SB maintain the capacity of surviving in the testis of mouse spermatogenesis deficiency models (3 out of 8), and no tumor structures was observed for at least two months through supervision of the recipient's seminiferous tubules and immunofluorescence staining (Fig. 6A,B). All these results indicated that mGSCs-I-SB maintain the mGSC unique characteristic of surviving in the receipt mouse spermatogenesis deficiency model testis.

THE CELLS CAN BE INDUCED TO DIFFERENTIATE INTO SPERM-LIKE CELLS

Male GSCs maintain the unique characteristic of differentiating into sperm cells [Olive and Cuzin, 2005]. To further demonstrate the immortalized cells maintain the capacity of mGSCs, the cells were

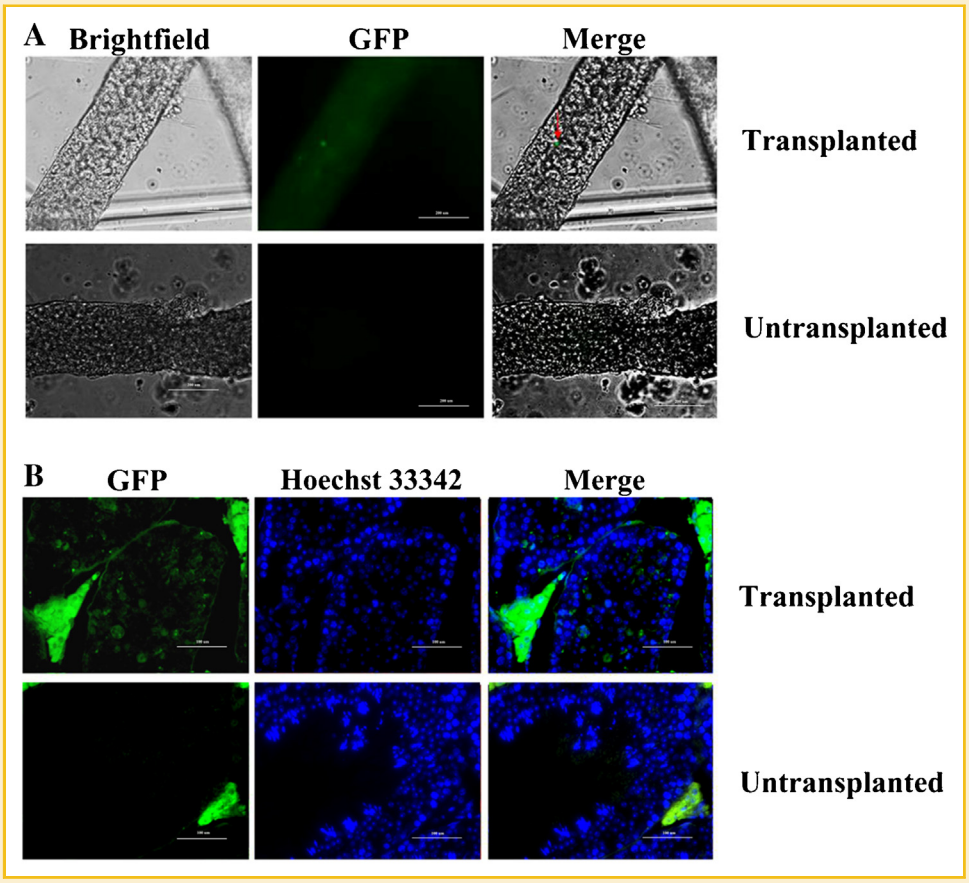

Fig. 6. Transplantation assay showed mGSCs-I-SB maintain the unique characteristic of mGSCs. After transplantation mGSCs-I-SB into receipt mouse spermatogenesis deficiency testis' seminiferous tubules for more than 2 months, the GFP-labeled cells were supervised in the seminiferous tubules (A) (Bar = 200 μm), and the transplanted mGSCs-I-SB cells existed and also were positive for GFP (B) (Bar = 100 μm).

cultured in suspension to form EBs (Fig. 7A). Then the EBs were induced under 2×10^{-7} μM RA. After 3 days induction, more round cells emerged in RA stimulation group than control; and this tendency seemed to be more obvious when the cells were induced for 7 days. Immunocytochemical staining was carried on to determine whether the induced cells maintain the characters of male germ cells. The results showed that the cells were positive for Stra8 (pre-meiosis marker), Scp3 (meiotic marker) and Acr (post-meiosis marker) (Fig. 7B). These results showed that mGSCs-I-SB maintain the unique capacity to differentiate into sperm-like cell (Fig. 7).

DISCUSSION

Previous reports on mGSCs were mainly in the mGSCs' establishment, growth, dedifferentiation, and transplantation [Olive and Cuzin, 2005]. All these progresses were mainly obtained on mouse and human. For other species, especially livestock, there is a lack of stable mGSC line. This greatly restricted the further study on the utilization of mGSCs in preservation and optimization of germplasm resources. Thus, the success in establishing immortalized dairy goat mGSC lines may set a model for other species' mGSCs.

Up to date, the immortalized germline cells including C-18-4, GC1, GC2, etc. were established by exogenous SV40T, hTERT, and/or BmI1 [Bellvé et al., 1977]. SV40 large T antigen, a hexamer protein that is a proto-oncogene derived from the polyoma virus SV40, is capable of transforming many cell types [Bellvé et al., 1977]. The transforming activity of T-tag is due in large part to its perturbation of the retinoblastoma (pRB) and p53 tumor suppressor proteins [Yang et al., 2007]. SV40 large T antigen has been shown to be the most simple and reliable agent for the transformation of many different cell types in culture. Most importantly, viral genes induce immortalization by inactivating the tumor suppressor genes (p53, Rb, and others) that can cause a replicative senescent state in cells. Recent studies have also shown that SV40 large T antigen can induce telomerase activity in the transduced cells. Additionally, T-tag binds to several other cellular factors, including the transcriptional co-activators p300 and CBP, which may contribute to its transformation function [Ali and DeCaprio, 2001]. BMI1 (BMI1 polycomb ring finger oncogene), a protein which in human is encoded by the BMI1 gene, has been reported as an oncogene by regulating p16 and p19 that are cell cycle inhibitor genes [Alkema et al., 1993]. Studies showed that inhibition of p16INK4a and introduction of hTERT can immortalize many types of human cells with little chromosomal instability [Haga et al., 2007].

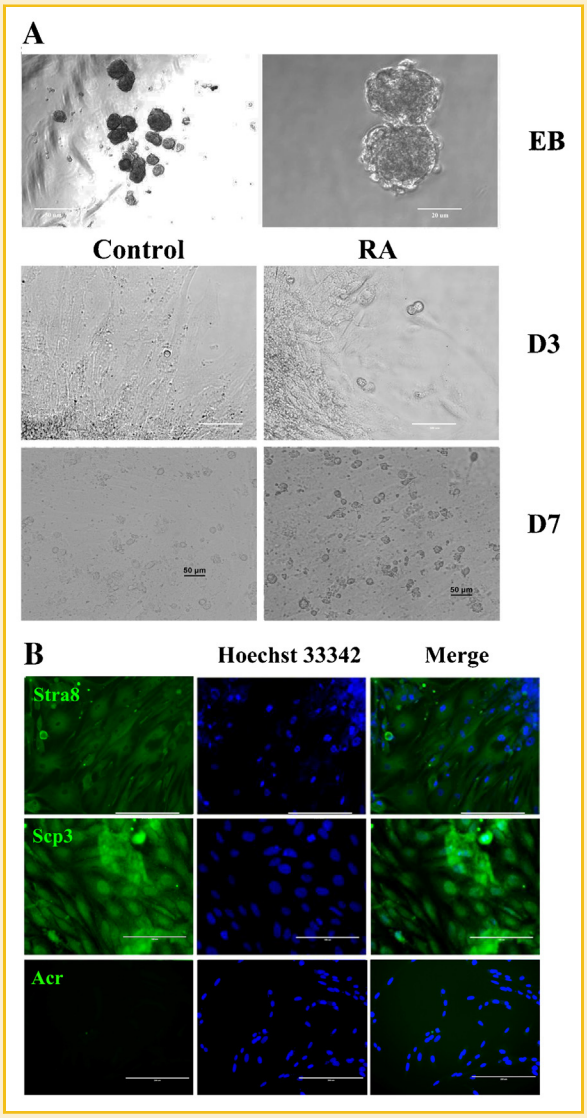

Fig. 7. The mGSCs-I-SB cells were differentiated into male germ cells in vitro. A: EBs were formed (from left to right, Bar = 50, 20 μm), mGSCs-I-SB were induced to form large, round cells by RA, also, the sperm-like cells were observed at 7 day induction by RA (D3, Bar = 100 μm; D7, Bar = 50 μm). B: Identification of the mGSCs-I-SB cells induced by RA through immunofluorescence staining. The induced mGSCs-I-SB cells were positive for STRA8, SCP3 (Bar = 100 μm) and ACR (Bar = 200 μm).

Lentiviral vector can be used to integrate both dividing and non-dividing cells as they can actively pass though nuclei membrane. In addition, same as retrovirus, lentivirus will integrate into a host cell genome. Lentivirus is gaining popularity for both in vitro and in vivo applications of gene transduction. In this study, our results for the first time showed that overexpression of ectopic factors-SV40 large T antigen and Bmi1 using lentivirus system may greatly improve the proliferation of dairy goat mGSCs analyzed by PDT, BrdU Incorporation assay and cell cycle. Meanwhile, the immortalized cells maintain the similar biological characteristics as wild mGSCs and previous reports on mouse mGSCs [Olive and Cuzin, 2005] when cultured the cells in similar conditions [Feng et al., 2002]. And, the cells expressed most of the conserved markers used in clarifying of mGSCs (Supplemented Figs. S2–S4). The immortalized cells also maintain the capacity to differentiate into all three germ layers as pluripotent mGSCs did as reported [Olive and Cuzin, 2005].

The golden standard method to examine the mGSCs is to transplant the cells into the mouse spermatogenesis deficiency model testis to determine whether the cells can reconstitute the seminiferous tubules which was established by Brinster and Avarbock [1994]. But this standard is mainly used in mouse and rat. For other species, whether the cells can survive for a long time is regularly evaluated [Olive and Cuzin, 2005]. In our study, the immortalized dairy goat mGSCs xenotransplantated into rodent model's seminiferous tubules and survived in recipient's testis for more than 2 months, and this greatly demonstrate the cells maintain the capabilities of mGSCs.

The chicken embryo is a classical animal model for studying embryonic and fetal development and for xenotransplantation experiments to study the behavior of cells in a standardized in vivo environment [Boulland et al., 2010]. Compared with the mammals, the main advantages of the chicken embryo are low cost, high accessibility, ease of surgical manipulation and lack of a fully developed immune system. Xenotransplantation into chicken embryos can provide valuable information about cell proliferation, differentiation and behavior, the responses of cells to signals in defined embryonic tissue niches, and tumorigenic potential [Boulland et al., 2010]. Transplanting cells into chicken embryos can also be a step towards transplantation experiments in other animal models. Recently the chicken embryo has been used to evaluate the neurogenic potential of human stem and progenitor cells following implantation into neural anlage [Boulland et al., 2010]. In this study, we transplanted the GFP-labeled mGSCs-I-SB into the developing central nervous system of the chicken embryo. The results evidenced that mGSCs-I-SB cells can survive, and meanwhile, and they differentiate into three germ layers *in* chicken embryos analyzed by immunofluorescence. This study first demonstrated chicken embryo was an efficient model to study the behavior and function of mGSCs.

Male GSCs have the ability to differentiate into spermatogenic cells through in vivo transplantation or through induction in vitro [Hua et al., 2011]. The immortalized mGSCs-I-SB were differentiated into sperm-like cells, and the induced cells were positive for Stra8 (a marker for pre-meiosis germ cells), Scp3 (a marker for meiosis cells) and Acr (a marker for post-meiosis cells). In combination with the transplantation assay, the results support that mGSCs-I-SB maintain the characteristic of mGSCs.

Taken together, our results for the first time demonstrate that the immortalized dairy goat mGSCs were obtained through exogenous expression of SV40 T antigen and Bmi1, and these cells have the characteristics of mGSCs compared with the mainly existed studies on mGSCs.

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