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Retinol (Vitamin A) Maintains Self-Renewal of Pluripotent Male Germline Stem Cells (mGSCs) From Adult Mouse Testis

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ABSTRACT

Studies have shown that male germline stem cells (mGSCs), which are responsible for maintaining spermatogenesis in the male, could be obtained from mouse and human testis. However, the traditional cultural methods were mostly dependent on serum and feeder, and the initial mGSCs were either obtained from neonatal mice or the detailed description of its potency and origin was not provided. Here we reported a novel (retinol (RE) serum-free and feeder-free) system for the successful culture of adult germline stem cells from adult Kunming mice (8–24 weeks) testis. The isolated mGSCs cultured in RE serum-free and feeder-free medium maintained the typical morphology of undifferentiated embryonic stem cells (ESCs), and they proliferated well in RE medium analyzed by proliferation assay, RT-PCR, microarray, and Western blotting. These cells also showed typical properties of ESCs (alkaline phosphatase (AP) positive, expressions of Oct4, Sox2, Nanog, and SSEA1, with the capacity to form teratomas and differentiate into various types of cells within three germ layers). Taken together, we conclude that RE promotes the self-renewal of mGSCs and maintains the pluripotency of mGSCs, the RE serum-free and feeder-free system may be useful for the culture of pluripotent stem cell lines from adult testis tissues, which provides a new resource for tissue engineering and therapy for infertility. J. Cell. Biochem. 112: 1009–1021, 2011.

KEY WORDS: MALE GERMLINE STEM CELLS (MGSCS); RETINOL (RE); SERUM-FREE AND FEEDER-FREE; GERM CELLS; EMBRYONIC STEM CELL (ESC); MOUSE

Spermatogenesis is a long process by which germline stem cells (GSCs) proliferate and differentiate into haploid sperm within seminiferous tubules of testes. As precursors of spermatogonial lineage, male GSCs maintain the balance of mature sperm production and self-renewal [Lee et al., 2006]. Since GSCs could renew themselves and produce spermatogenic cells after transplantation into the seminiferous tubules of infertile recipient males, they constitute a model system for understanding spermatogenesis and developing a novel transgenic approach [Brinster and Zimmermann, 1994; Brinster, 2002].

Recent studies demonstrated the establishment of germline stem cell line derived from both neonatal and adult male testis, and these cells showed similar properties to ESCs [Kanatsu-Shinohara et al., 2003a,b, 2005, 2008; Guan et al., 2006, 2009; Lee et al., 2006; Conrad et al., 2008; Mizrak et al., 2009]. However, GSCs are rare in testis, constituting ~1 in 3,000–4,000 cells in mouse testis [Telgelenbosch and de Rooij, 1993], and it seems to be impossible to establish pluripotent cells from some strains [Kanatsu-Shinohara et al., 2004]. Additionally, mouse GSCs were usually generated in vitro by genetic modification or co-culturing with feeder cells [Feng et al., 2002; Kanatsu-Shinohara et al., 2003b; Lee et al., 2006], though scientists have also reported to culture GSCs in serum- and feeder-free system, the media were complex and expensive (Kanatsu-Shinohara et al., 2005; Lim et al., 2010).

Shanshan Zhang, Junwei Sun and Shaohui Pan contributed equally to this work.

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Generally, serum and feeder layers are essential for proliferation and maintenance of ESCs [Guan et al., 2006, 2009; Kanatsu-Shinohara et al., 2006]. Fetal bovine serum (FBS), as a source of growth factors, is usually added to media for ESC cultures [Cheng et al., 2004]. Many proteins and growth factors in serum play important roles in maintaining the undifferentiated state of ESCs. However, some factors in FBS and feeder cells also promote the differentiation of ESCs, which may make it difficult to establish and maintain them in an undifferentiated state [Hua et al., 2009]. Previous reports showed that glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF) and other cytokines play important roles in maintaining GSC proliferation in vitro [Kanatsu-Shinohara et al., 2003a,b, 2005, 2008; Caires et al., 2010]. Retinol (RE), the alcohol form of vitamin A, maintains pluripotency of ESCs by over-expression of Nanog, which is a key transcription factor for self-renewal, and RE could support the self-renewal of ESCs in feeder-free condition without affecting their pluripotency [Chen et al., 2007; Chen and Khillan, 2008, 2010]. ESGRO Complete Clonal Grade Medium (Clonal medium) is a defined serum-free medium optimized to culture and maintain undifferentiated mouse ESCs in the absence of serum and feeder cells [Banerjeea and Bacanamwo, 2010]. However, whether the serum-free and feeder-free medium is suitable for the proliferation of Kunming mice GSCs has not been investigated in particular [Buehr and Smith, 2003].

In this study, we first described the derivation of mGSCs from the adult Kunming mouse testis by using different medium, and then compared the efficiencies of forming AP positive colonies. Results showed that ESGRO complete^{TM} medium for mouse embryonic stem cell (ESC) culture, or knockout serum replacement (KSR) medium supplemented with LIF or RE could maintain the undifferentiated state of GSCs well and we obtained mouse GSCs line. Then we subcultured the GSCs in RE serum-free and feeder-free medium sequentially for more than 10 passages, and they still shared the characteristics of pluripotent markers analyzed by RT-PCR, immunofluorescence and microarray. Also, we found that these cells had the capacity to differentiate into various types of cells including all three germ layers, especially sperm-like cells in vitro. This will provide us an efficient system to culture pluripotent stem cell lines and study the origin and specification of germ cells.

MATERIALS AND METHODS

Kunming strain mice (aged = 8–24 weeks; body weight = 30–40 g), were used throughout the study. They were maintained under standard conditions with free access to food and water at the Animal Facilities in our lab. All of the feeding and experimental procedures on animals were in accordance with the guidelines of the local laws.

ISOLATION OF MOUSE mGSCs

Testicular tissues obtained from adult Kunming mice were incubated in 2 ml of enzyme solution containing 1 mg/ml collagenase (Type I; Sigma, St. Louis, MO), 10 μg/ml DNase I and 1 mg/ml hyaluronidase (Sigma) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) for 30 min at 37°C for dissociation [Pan et al., 2010]. The primary cells were seeded as 1×10^5/ml onto mitotically inactivated mouse embryonic fibroblast (MEF) cell feeders (5×10^4 cells/cm^2) as described. Then the cells were maintained at 37°C in a humidified 5%CO_2, 95% air atmosphere. After 1–2 weeks, large multicellular (>100 cells) colonies that formed on the dish were sequentially passaged every 3 days. The medium was changed every 2 days and cells were subcultured with Tryple or Accutase (Invitrogen, Carlsbad, CA) at a 1:2–6 dilution.

To evaluate the effects of different cytokines on the formation of AP positive colonies for the primary culture of mGSCs, the culture media for GSCs consisted of serum-free medium (ESGRO complete^{TM}, Clonal medium, Chemicon, Billenca, MA, CA) and the normal medium consisted of Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 15% KSR (Invitrogen) or 15% FBS (Hyclone, Logan, UT), 0.1 mM 2-mercaptoethanol (Invitrogen), 4 mM glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen). Human GDNF (Peprotech, NJ), human LIF (Millipore), human bFGF (Millipore) and RE (Sigma) were used in the primary GSC cultures at a final concentration of 1,000 IU/ml, 10 ng/ml, 10 ng/ml, and 5×10^{-6} M, respectively. The primary cells were cultured as 1×10^4 /ml (96-well plate). The number of AP positive colonies was counted at 3, 7, and 10 days after culture.

Then, the mGSCs colonies in Clonal medium were dissociated mechanically or by Tryple. During the primary culture, we discovered that mGSCs could be obtained by serum-free and feeder-free condition, and RE could also promote proliferation of mGSC to some extent. Therefore the subcultured mGSCs were cultured in serum-free and feeder-free system including (1) KSR (control) medium: DMEM containing 15% KSR (Invitrogen), 4 mM glutamine, 0.1 mM 2-mercaptoethanol (Invitrogen), 1% non-essential amino acids (Invitrogen); (2) RE medium: KSR medium supplemented with 5×10^{-6} M RE (Sigma, CA); (3) LIF medium: KSR medium supplemented with 1,000 IU/ml LIF. After 48 h, we counted the total number of mGSCs in different media and calculated cell population doubling times (PDT). PDT were calculated from the formula, $PDT = [\log 2/(\log N_t - \log N_0)] \times t$, where N_t = number of cells after t hours of culturing, N_0 = number of cells seeded, r/day = rate of cell proliferation per day [Jung et al., 2010].

CHARACTERIZATION OF mGSCs CULTURED IN RE SERUM-FREE AND FEEDER-FREE SYSTEM

GROWTH CURVE

The mGSCs were seeded to 24-well plates at 1×10^4 cells/well and cultured in RE serum-free and feeder-free medium. The proliferation ability was assessed by growth curve at an interval of 24 h, respectively. The cells were trypsinized and cell numbers were determined for 4 consecutive days (n = 3).

AP STAINING

AP activity was determined essentially as described by Piedrahita et al. [1998]. Briefly, culture plates were rinsed three times in PBS and fixed in 4% paraformaldehyde (PFA) for 10–15 min at room temperature. Fixed cells were washed three times with PBS and stained with naphthol AS-MX phosphate (200 μg/ml, Sigma) and

TABLE I. The Primer Sequences and PCR Reaction Conditions

Name	Sense primer	Antisense primer	Product size	T_m (°C)
β-actin	ACGGCATCGTCACCAACT	AGGAAGGAAGGCTGGAAGAG	583	58
Oct4	CGCCCGCATACGAGTTCT	GCACCAGGGTCTCCGATTT	487	58
Sox2	GCCCAGGAGAACCCCAAGAT	GGGTGCCCTGCTGCGAGTA	520	54
Nanog	GATTCTTCTACCAGTCCCAAAC	ATGCGTTCACCAGATAGCC	376	54
C-kit	TATGATCACAAATGGGAGTTTCC	TTCTTATAAAGTGCCGCTTCTG	383	57
Vasa	AAAGTGCCCAGTTCTTGTTGCTAC	ACTGGATTGGGAGCTTGTGAAG	392	58
CDK2	GCCAGGAGTTACTTCTATGC	TGGAAGAAAGGGTGAGCC	180	53
Cyclin D1	TGAACTACCTGGACCGCT	CAGGTTCCACTTGAGYTTGT	212	50
Cyclin A	TGGCTGTGAACTACATTGA	ACAAACTCTGCTACTTCTGG	136	50
Fgf-5	CCTTGCTCTTCCTCATCTTCTGC	GAGCCATTGACTTTGCCATCCG	319	58
AFP	ATCCTCCTGCTACATTTCGCTGC	TGAGCAGCCAAGGACAGAATG	512	58
Nf-68	TTCTCCCCCGTTCTTCTCTCTAG	CTTCTCGTTAGTGGCGTCTTCC	540	58
Brachury	AAGGTGGCTGTTGGGTAGGGAGT	ATTGGGCGAGTCTGGGTGGATGT	451	58
TTR	ACTCTTCCTCCTTTGCCTCGCTG	GCAGGGGAGAAAAATGAGGAAAT	592	58
Gdf-3	CCTTATCAACGGCTTCTGGCGC	CTCTAAGTGTAAGTCCAAGT	606	58
Klf4	CCAGGAGAACCCCAAGATGC	GGGTGCCCTGCTGCGAGTA	518	58
C-Myc	CTGGTGGGCGAGATCATCA	CACTGCCATGAATGATGTTCC	304	54

Fast Red TR salt (1 mg/ml, Sigma) in 100 mM Tris buffer, pH 8.2–8.4, for 10–30 min at room temperature, and washed with PBS to terminate staining.

FLUORESCENCE STAINING

Colonies of putative male GSCs were fixed in 4% PFA for nucleus counting using 1 μg/ml Hoesch33342 (Sigma). Moreover, fixed cultures were incubated overnight with the specific monoclonal or polyclonal antibodies of Oct-4 (1:200, Polyclonal, Chemicon), Sox2 (1:200, Polyclonal, Chemicon), Nanog (1:200, Polyclonal, Chemicon), CD49f (1:500, Monoclonal, Chemicon), SSEA1 (1:200, Monoclonal, Chemicon) and Vasa (1:200, Polyclonal, Abcam), which are markers expressed in GSCs. The neural cell, cardiomyocyte and sperm-like cell differentiation were evaluated by NSE-neuron-specific enolase (1:200, Polyclonal, Millipore), β-III tubulin (1:1,000, Monoclonal, DSHB, Iowa City, IA), cardiac α-actin (1:500, Monoclonal, Sigma), Troponin T-C (CT3, 1:1,000, DSHB), Synaptonemal Complex Protein 3 (Scp3, 1:300, Polyclonal, Santa Cruz Biotechnology, Inc., CA), β1-integrin (1:100, Monoclonal, Chemicon), a surface marker of primordial germ cell (EMA1, 1:100, DSHB) and an antibody for the sperm acrosome (FE-J1, 1:100, Monoclonal, DSHB) antibodies. The primary antibodies were visualized with fluoroscein isothiocyanate or Cy3-conjugated secondary antibody (1:500, Polyclonal, Millipore).

RT-PCR

RT-PCR was performed to assess the expression of stage-specific marker genes in mGSCs cultured in KSR, RE, LIF, and Clonal medium at 48 h after subculture. Pluripotent markers-Oct4, Sox2, C-myc, Nanog, and genes related with proliferation and cell cycle such as proliferating cell nuclear antigen (PCNA), cyclin A, cyclin D1, and CDK2 were analyzed. mGSC markers including Oct4, Sox2, Nanog, Stella, Dazl, and Vasa were also analyzed in typical mGSCs colonies (P8) cultured in RE serum-free and feeder-free medium, ESC (J1), embryonic bodies derived from mGSCs (d3) and adult testis. β-actin was used as internal control.

Total RNA was extracted by using the Trizol method (Invitrogen). The cDNA was synthesized based on 500 μg RNA with a commercially available kit (Fermentas). Amplification was performed in a 15 μl reaction mixture containing 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 0.25 mM dNTP, 3 pM of each primer and 1.25 IU Taq polymerase (Invitrogen). The following genes were amplified using the primers indicated in Table I.

PCR was initiated with denaturation at 94°C for 5 min, followed by 30–35 cycles of 30 s at 94°C, 30 s at 55–60°C, and 30 s at 72°C, with a final extension for 10 min at 72°C. The products were separated by 2% agarose gel electrophoresis and verified.

MICROARRAY ANALYSIS

The microarray study was carried out using either Mouse Genome Oligo GeneChip arrays (SBC, Shbioship, Shanghai). The protocol was based on manufacture's manual. The total RNAs of mGSCs (P14) cultured in RE serum-free and feeder-free medium and ESCs (J1) were used for microarray analysis. *t*-Test was used to analyze the data. If the value >2 or <0.5, the gene was believed as up-regulated or down-regulated.

WESTERN BLOTTING

Total cell extracts were prepared from mGSCs cultured in KSR, RE, LIF, and Clonal medium after subculturing for 48 h. Total cell proteins were resolved by 10% SDS–PAGE, transferred to PVDF membrane, and probed with β-actin (1:1,000, Beyotime, Haimen, Jiangsu, China), PCNA (1:1,000, Millipore), C-Myc (1:1,000, Chemicon). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as a secondary antibody (1:1,000, Beyotime). The detection was performed followed by visualization using the BM-Chemiluminescence blotting substrate (Roche, Shanghai, China).

RETINOL MAINTAINS DIFFERENTIATION POTENTIAL OF mGSCs

TERATOMA FORMATION

mGSCs colonies were cultured in RE-medium consecutively for at least 24 days and dispersed into single cells by incubation in Tryple for 2–3 min at 37°C. Nude mice were injected subcutaneously using a 30 gauge needle with 2×10^6 mGSCs. Tumor formation was identified by manually palpating the injected area from 7 days after

injection. After 35 days of growth, tumor tissue was removed, fixed in 4% PFA, and processed for paraffin sectioning.

NEURAL LINEAGE DIFFERENTIATION

For 3 days in hanging drops, EBs were placed in suspension culture in bacteriological petri dishes with DMEM containing 15% FBS, 0.1 mM β-mercaptoethanol and 10^{-5} M all-trans retinoic acid (RA) (Sigma-Aldrich) for 3 days. Then differentiating EBs were placed in 24-well plates in the presence of DMEM/F12, supplemented with 15% FBS, 10 ng/ml bFGF, 20 ng/ml EGF (epidermal growth factor, Sigma-Aldrich) and the RA was omitted from the culture 2 days after plating. The cells were cultured for an additional 2 weeks and the neuronal differentiation was verified and characterized by fluorescence analysis.

CARDIOMYOCYTE DIFFERENTIATION

EBs were transferred from hanging drops after 3 days of suspension culture into bacteriological petri dishes, with DMEM containing 15% FBS, 10 ng/ml bone morphogenetic protein 4 (BMP4) or 10 μM 5-aza-2′-deoxycytidine (5-aza) for 7 days (BMP4) or 2 days (5-aza) respectively. At day 11, EBs were transferred onto coverslips and placed into individual wells of a 48-well plate, followed by incubation for 10 more days (22 days of differentiation totally) in DMEM plus 20% FBS.

PRODUCTION OF HAPLOID MALE GERM CELLS

The 8–12th passage mGSCs were used to induce differentiation. mGSC colonies and EBs derived from mGSCs were trypsinized into single cells, resuspended and transferred into culture medium consisting of DMEM (Invitrogen) supplemented with 5 μg/ml insulin-transferring-selenium solution (Invitrogen), 2×10^{-6} M retinoic acid (Sigma), 1 mM pyruvate (Sigma), 1× antibiotic–antimycotic (containing penicillin, streptomycin, and amphotericin B; Invitrogen), and 15% FBS (Hyclone). Some EBs were induced by 10^{-6} M retinoic acid (RA, Sigma, USA) and follicle stimulating hormone (FSH, 0.5 U/ml, Ningbo Biomedical Factory, Ningbo, China) in DMEM and the cells were cultured for 10–20 days in this medium combined with extracts from adult goat testis (GE). The preparation of testis extracts was based on Håkelien et al. [2006]. Protein concentration of the extracts was estimated spectrophotometrically and added in the medium (50 μg/ml).

RESULTS

GENERATION OF PLURIPOTENT MOUSE ADULT mGSCs

We used 13 different adult mouse testicular tissues in total to generate mGSCs. The obtained tissues were mechanically and enzymatically dissociated and filtered to obtain single-cell suspensions containing cells of varying sizes and shapes. Most cells were cultured in gelatin-coated dishes for 12–24 h, and then we removed the suspended cells onto MEF feeder layers or cultured them in Clonal medium. The cells formed mouse ES-like colonies in an undifferentiated state after 4–7 days (Fig. 1A,B). GSC-like colonies were obtained from testes of 10 adult Kunming mice (8–24 weeks) in the commercial serum-free media, ESGRO complete™ (Clonal medium). With this selection procedure we were able to obtain a pure population of mGSCs. After the selection and purification, colonies of mGSCs appeared and increased in size. After 10–15 days, these colonies changed their morphology and became multilayered and clearly demarcated colonies with

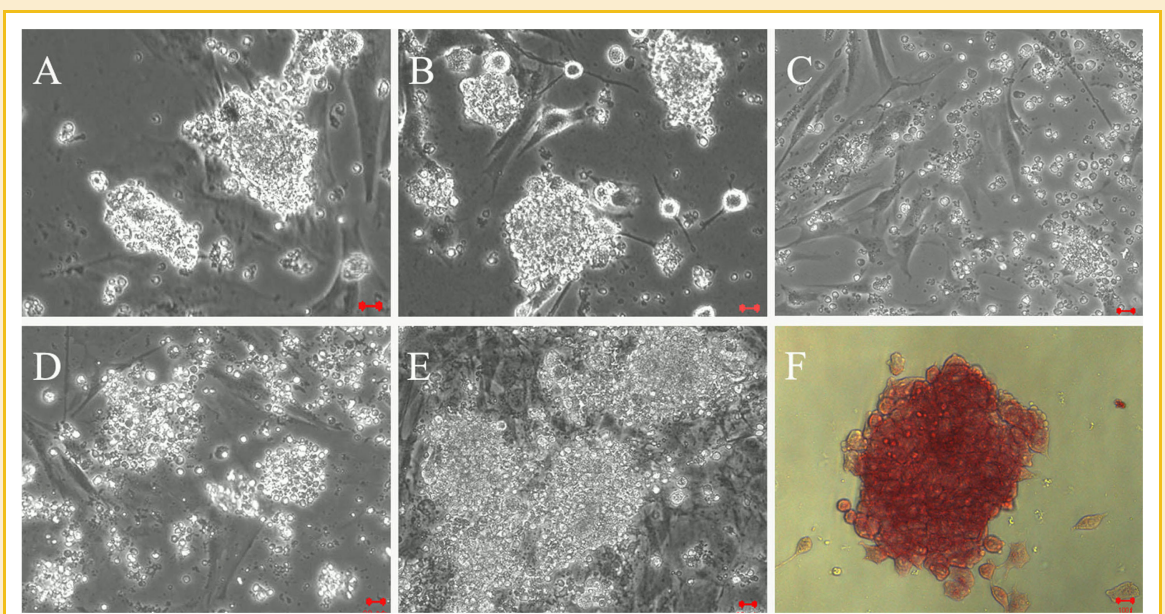

Fig. 1. The morphology of mGSC colonies. A,B: The spermatogonial cells formed small ES-like mGSC colonies on MEF feeder after selection culture. (A) Bar = 20 μm, (B) bar = 20 μm. C–E: The putative ES-like mGSC colonies formed in serum-free and feeder-free Clonal medium. Bar = 20 μm. F: The typical mGSC colonies cultured in RE serum-free and feeder-free medium were positive for AP. Bar = 20 μm.

boundaries appeared (Fig. 1C). These colonies continued to increase in number and size (Fig. 1D). After 2–4 weeks, these colonies continued to proliferate successfully and these adult mGSCs had a stable, stem cell-like phenotype (Fig. 1E), which were positive for AP (Fig. 1F).

In the primary cultures, we compared the effects of different cytokines and factors including RE, LIF, bFGF, GDNF, FBS, KSR, and Clonal medium on the proliferation of AP positive colonies. We found that Clonal medium was the most efficient medium to obtain AP positive mGSC colonies. LIF is essential for the isolation of AP positive GSC colonies. Also, RE can replace LIF to some extent and improve the formation of AP positive GSC colonies. KSR serum-free medium was more efficient in isolation of AP positive GSC colonies than FBS medium. GDNF improves the colonization of AP positive mGSC colonies derived from adult mouse testis. However, bFGF was not beneficial for the obtaining of AP positive mGSC colonies (Fig. 2).

RETINOL SUPPORTS SELF-RENEWAL OF mGSCs

These cells formed typical GSC colonies and the number of cells cultured in RE medium was significantly more than KSR control, which is similar to medium containing LIF ($P < 0.05$, Fig. 3A–E). There were significant differences in PDT among cells cultured in RE and LIF in KSR medium (Table II, $P < 0.05$), and the number of AP positive mGSC colonies in RE medium was more than KSR medium (Fig. 3F–G, $P < 0.05$). The results showed that mGSCs proliferated faster in RE medium than that in KSR control medium and mGSCs had strong proliferation in colonization in the RE serum-free and feeder-free system.

RT-PCR analysis showed that expressions of PCNA, C-myc, cyclin D1, CDK2, and cyclin A, which were markers of cells indicating strong proliferation, were up-regulated compared to KSR control medium (Fig. 3H). Simultaneously, they maintained the pluripotent markers being similar to those cultured in commercial Clonal and LIF medium. Western blotting also showed that the expression of PCNA and C-myc protein were higher in mGSCs cultured in RE medium than that in KSR control (Fig. 3I).

PLURIPOTENCY OF ADULT MOUSE mGSCs CULTURED IN RE SERUM-FREE KSR MEDIUM

The ES-like colonies derived from adult mouse testis maintained a stable proliferation in RE serum-free and feeder-free system (Fig. 4A). The cells were cultured for 18 passages in RE serum-free medium and passaged every 2–3 days, these ES-like colonies (Fig. 4B–E) also highly expressed AP (Fig. 4F), and pluripotent transcription factors of ESCs even in higher passages, such as

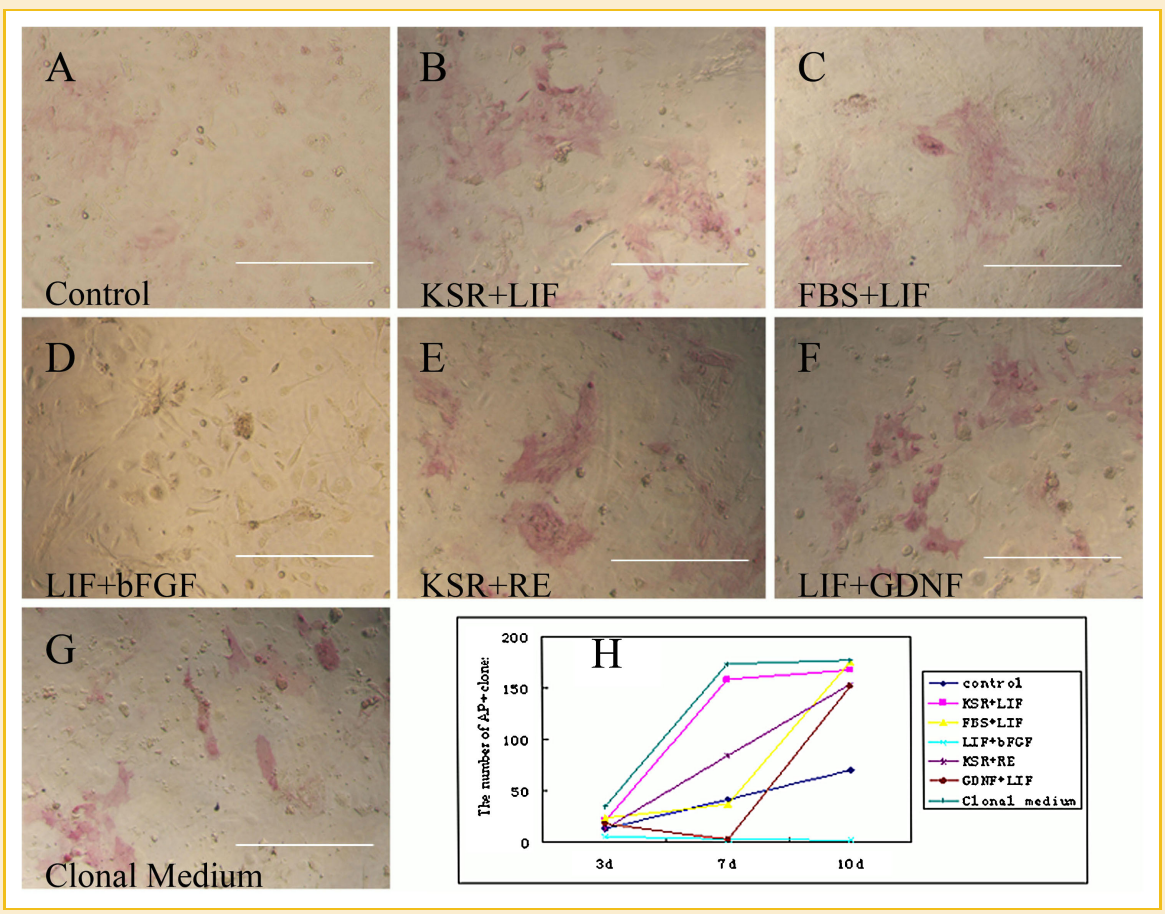

Fig. 2. Morphology and AP staining of primary GSCs cultured in different medium and the effects of different cytokines on the formation of AP positive GSC colonies. Bar = 200 μm.

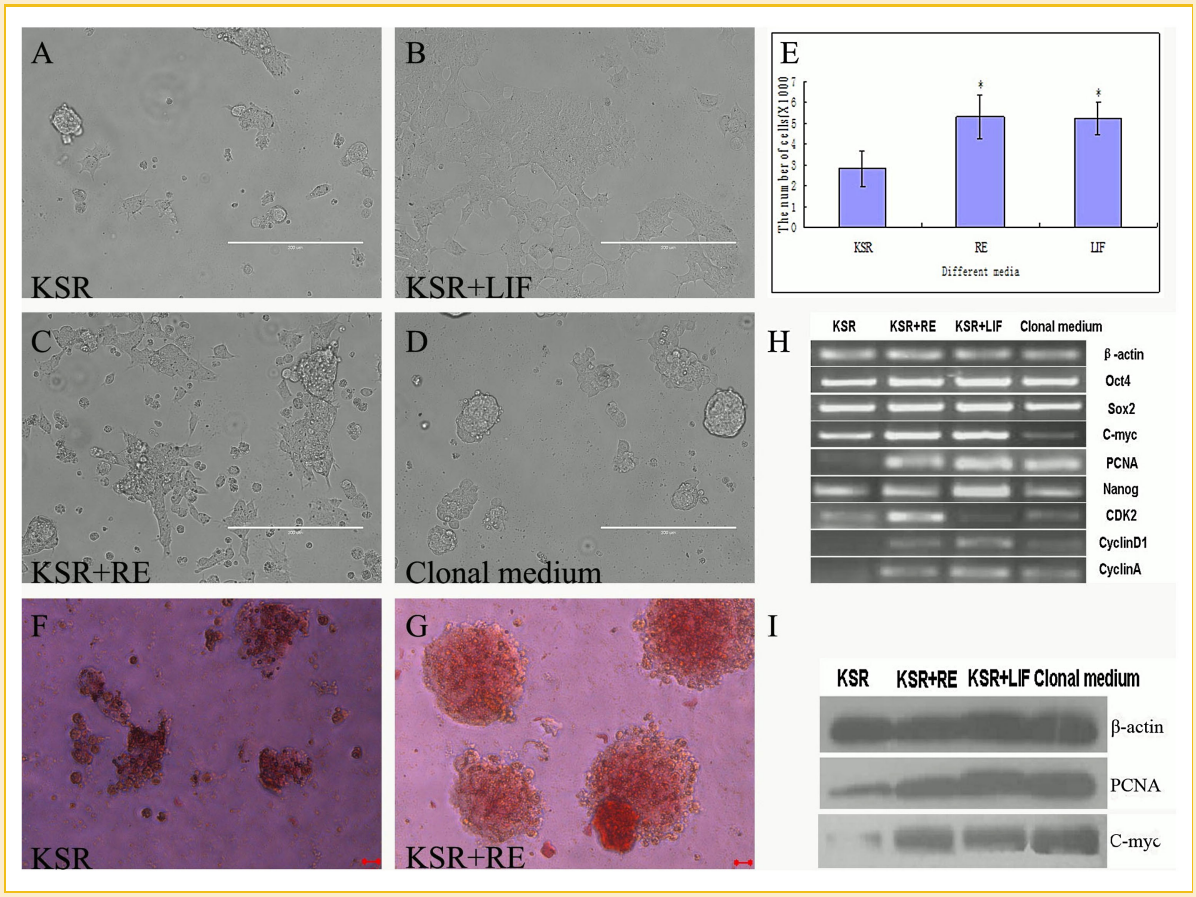

Fig. 3. Characterization of mGSC cultured in RE serum-free and feeder-free medium. The proliferation potential of GSC colonies cultured in RE medium was obviously stronger than KSR control medium, and the number of cells was significantly more than KSR control, which is similar to medium containing LIF ($P < 0.05$, A–E). Bar = 200 μm (A–D). The number of AP positive GSCs colonies in RE medium was more than KSR medium (F,G, $P < 0.05$). Bar = 20 μm (F,G). H: RT-PCR analysis showed that expressions of PCNA, C-myc, cyclin D1, and cyclin A were up-regulated compared to KSR control, which were markers of cells with strong proliferation (H). The pluripotent markers are similar as GSCs cultured in Clonal and LIF medium. I: Western blotting analysis showed that the expression of PCNA and C-myc was increased in GSCs cultured in RE medium than that in KSR control.

Oct3/4, Nanog, and Sox2 (Fig. 4G–I). The generated adult mouse mGSC colonies were also positive for SSEA1 (a ESC marker, Fig. 4J), CD49f and Vasa (markers of mGSCs, Fig. 4K,L). These results indicate that the mouse mGSCs express the surface markers and the transcription factors, which are characteristics of undifferentiated ESCs and typical mGSCs [Guan et al., 2006; Conrad et al., 2008].

To confirm our data, we examined the expression of genes specific to ESCs and mGSCs cultured in RE serum-free KSR medium by RT-PCR and microarray analysis. Results demonstrated that our cultured adult mGSCs expressed both ES cell markers such as Oct3/4, Nanog, Sox2 and mGSC markers like Stella, Dazl, and Vasa (Fig. 4M). Furthermore, global gene expression of GSCs and ESCs was compared by hierarchical cluster analysis, the scatter plots of microarray analyses highlighted the differences between mGSCs and ESCs and demonstrated the similarity between mGSCs and ESCs (Fig. 4N). Taken together, these results demonstrate that the cellular and molecular characteristics of the mGSCs are very similar to those of ESCs. Thus, the mouse mGSC colonies derived from testis germ cells present showed relatively high levels of ESC markers [Guan et al., 2009]. These results indicate that mouse adult mGSCs respond to culture conditions and could maintain mouse ESC properties in RE serum-free and feeder-free system.

TABLE II. The PDT of mGSCs Cultured in RE Serum- and Feeder-Free Medium

Groups	PDT (h)	r/day
KSR	34.71 ± 0.31	0.78 ± 0.20
RE	$22.73 \pm 0.22^{*}$	$1.16 \pm 0.14^{*}$
LIF	$21.60 \pm 0.12^{*}$	$1.19 \pm 0.09^{*}$

*Significant differences ($P < 0.01$).

mGSCs CULTURED IN THE RE SERUM-FREE AND FEEDER-FREE SYSTEM FORM TERATOMAS IN VIVO AND MAINTAIN DIFFERENTIATION POTENTIAL IN VITRO

Adult mouse mGSCs in the RE serum-free and feeder-free system were also examined for their pluripotency in forming teratomas in vivo after injection into immunodeficient mice. Teratomas formed after 35 days of growth (Fig. 5A). The outgrowth of the teratomas contained cell types including derivatives of endodermal, meso-

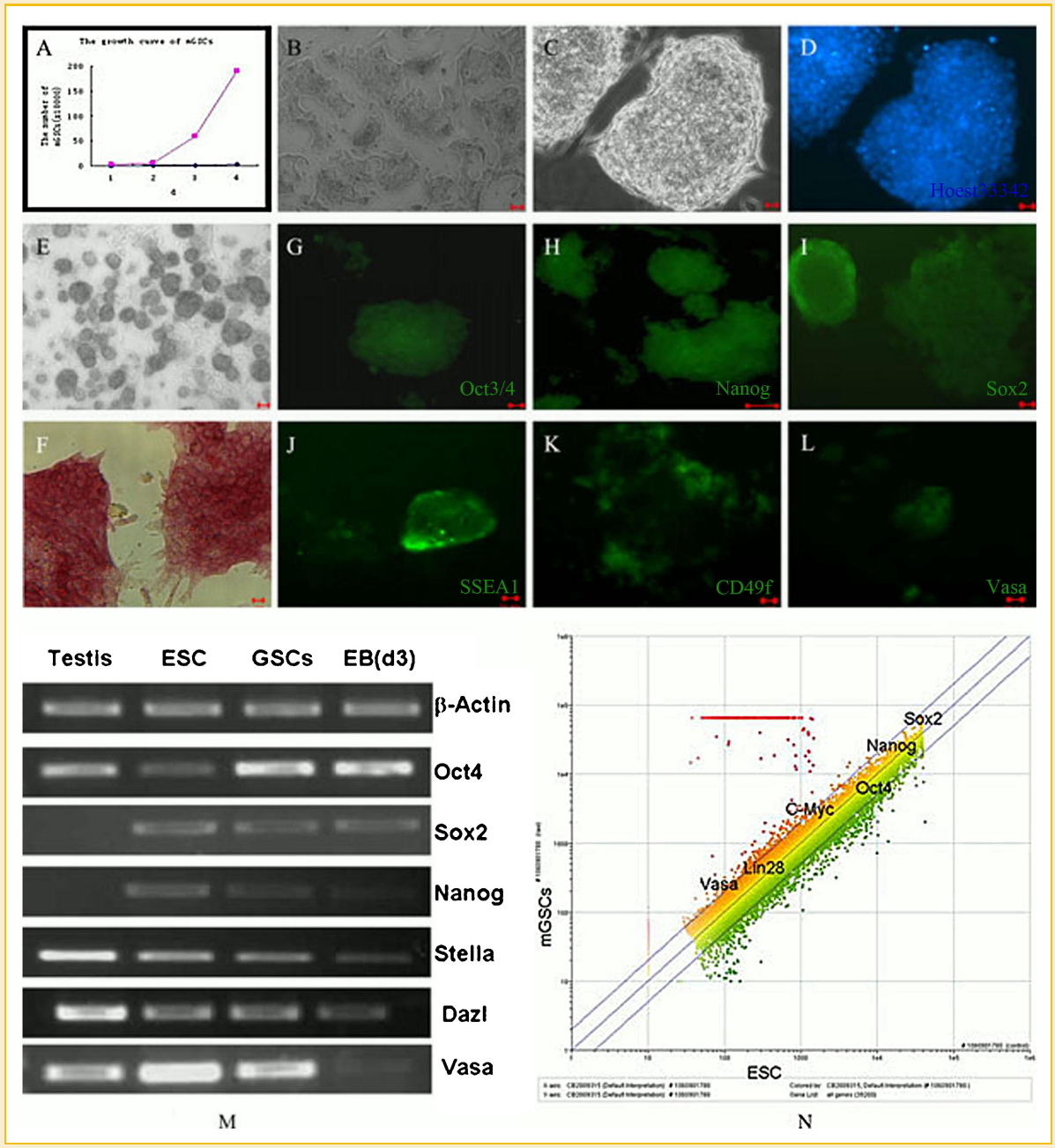

Fig. 4. ES cell-like mGSC colonies formed in RE serum-free and feeder-free system. A: Growth curve of mGSC. B: mGSC colonies (passage 8) in RE serum-free and feeder-free system, bar = 100 μm. C: Passage 11 mGSC colonies, bar = 20 μm. D: DAPI labeled the nuclei of passage 11 mGSC, bar = 20 μm. E: Passage 14 mGSC colonies, bar = 100 μm. F: mGSCs were positive for AP, bar = 20 μm. G: mGSCs were positive for Oct4, bar = 20 μm. H: mGSCs were positive for Nanog, bar = 50 μm. I: mGSCs were positive for Sox2, bar = 20 μm. J: SSEA1, bar = 20 μm. K: CD49f, bar = 20 μm. L: Vasa, bar = 20 μm. M: RT-PCR analysis of mGSCs cultured in RE serum-free and feeder-free medium. Mouse mGSCs expressed ES cells and germ cell markers such as Oct4, Sox2, Nanog, Stella, Dazl, Vasa analyzed by RT-PCR. The expression profile of mGSCs is similar as ESCs, and the pluripotent markers were down-regulated in EBs (d3). N: Microarray analysis showed that mGSCs cultured in RE serum-free and feeder-free medium expressed pluripotent ESCs markers including Oct4, Sox2, Nanog, C-myc, Klf4, Nanog, and Lin28.

dermal, and ectodermal embryonic germ layers: stratified cell epithelium, neuronal cells, cartilage, muscle, glandular structures, and endodermal high prismatic epithelium (Fig. 5B–F).

To determine whether mouse adult mGSCs can differentiate in vitro, we applied common methods designed to induce differentiation of mouse mGSCs into neural cells, cardiomyocytes, and sperm lineages [Golestaneh et al., 2009]. On the whole, adult mGSCs formed typical embryoid bodies (EBs) in suspension culture (Fig. 5A′,B′). Then the EBs were attached to dishes to differentiate into many cell types within three germ layers. After 13 days, Nestin positive neural-like cells (ectoderm) (Fig. 5C′), AFP (an endoderm marker, Fig. 5D′) positive, and Oil red O positive adipocyte-like cells (mesoderm) were produced (Fig. 5E′). RT-PCR analysis also demonstrated that EBs (day 7) derived from mGSCs expressed

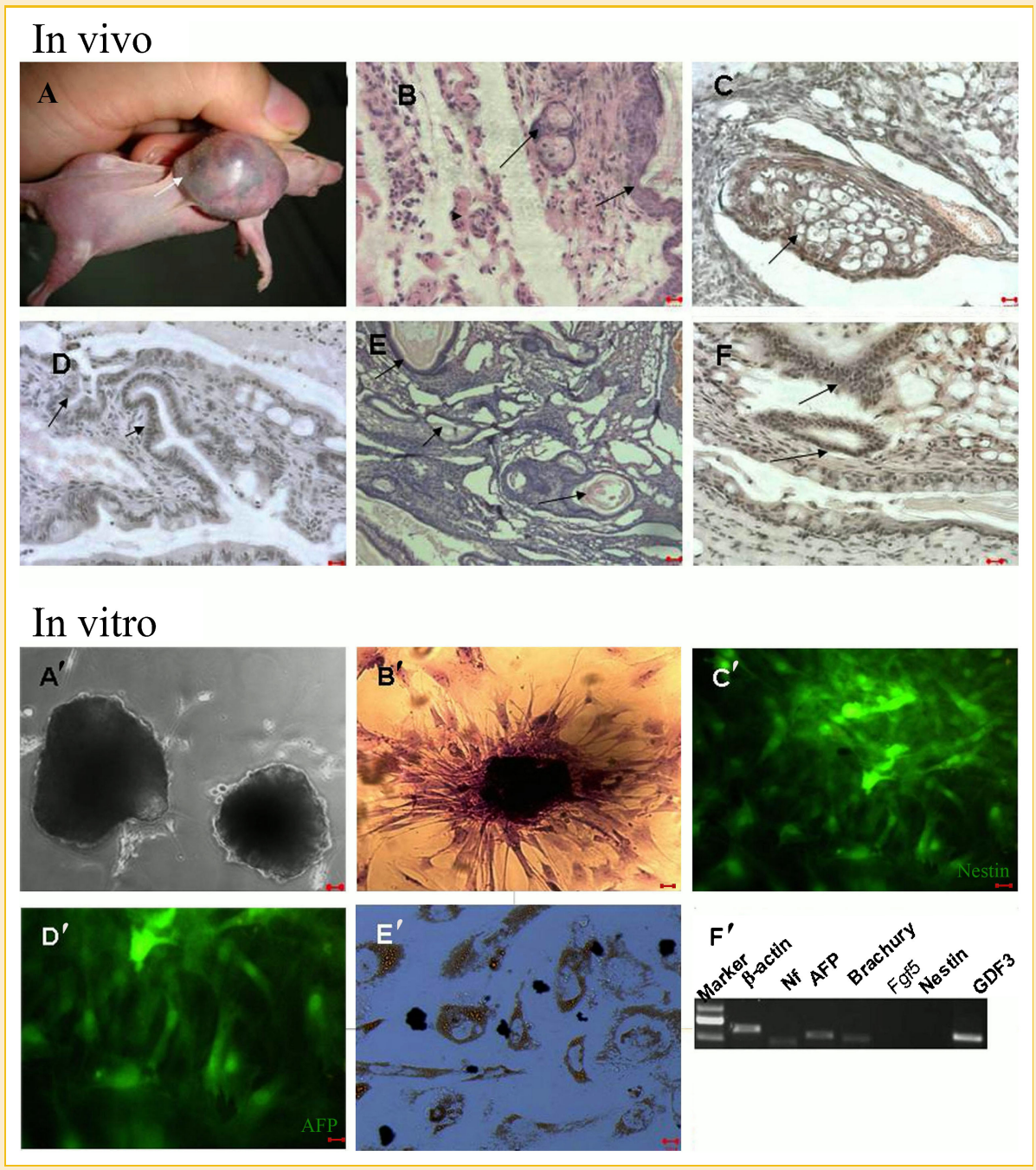

Fig. 5. Differentiation of mGSCs in vivo and in vitro. In vivo: the teratoma (A) was shown to contain tissues representative of all three germ layers. Muscle and cartilages (B,C), Intestine (D), respiratory epithelial (E) and neural tissue (F). Bar = 20 μm. In vitro: A′, embryoid bodies (EBs) derived from mGSCs; B′, giemsa staining of EBs; C′,D′, plated EBs derived from mGSCs differentiated into AFP and β-III tubulin positive cells; E′, adipocyte like cells were produced from mGSCs, which were positive for Oil red O, bar = 20 μm (A′–E′). F′: RT-PCR analysis demonstrated that EBs (day 7) derived from mGSCs expressed the markers of three germ layers. β-actin as internal control, TTR (ectoferm), Nf-68 (ectoferm), AFP (endoderm), Bra (mesoderm) were expressed in EBs (day 7) derived from mGSCs, Fgf-5 and Gdf3 were not expressed.

markers of three germ layers such as a 68-kDa neuro-filament marker (Nf-68, ectoderm), transthyretin (TTR, endoderm), alpha-fetoprotein (AFP, endoderm), Brachury (Bra, mesoderm). However, fibroblast growth factor 5 (Fgf-5, ectoderm) and growth differentiation factor-3 (Gdf3, pluripotent marker) was not detected at mRNA level (Fig. 5F′).

Cardiomyocytes and neural cells differentiation displayed cardiac and neural morphological characteristics after induction. Neuron like cells and astrocytes were positive for neuron-specific enolase (NSE) and β-III tubulin in RA-treated cultures. Also typical beating cardiac cell clusters appeared in BMP4-induced cultures and these cells expressed cardiac muscle specific α-actin and CT3 (Fig. 6).

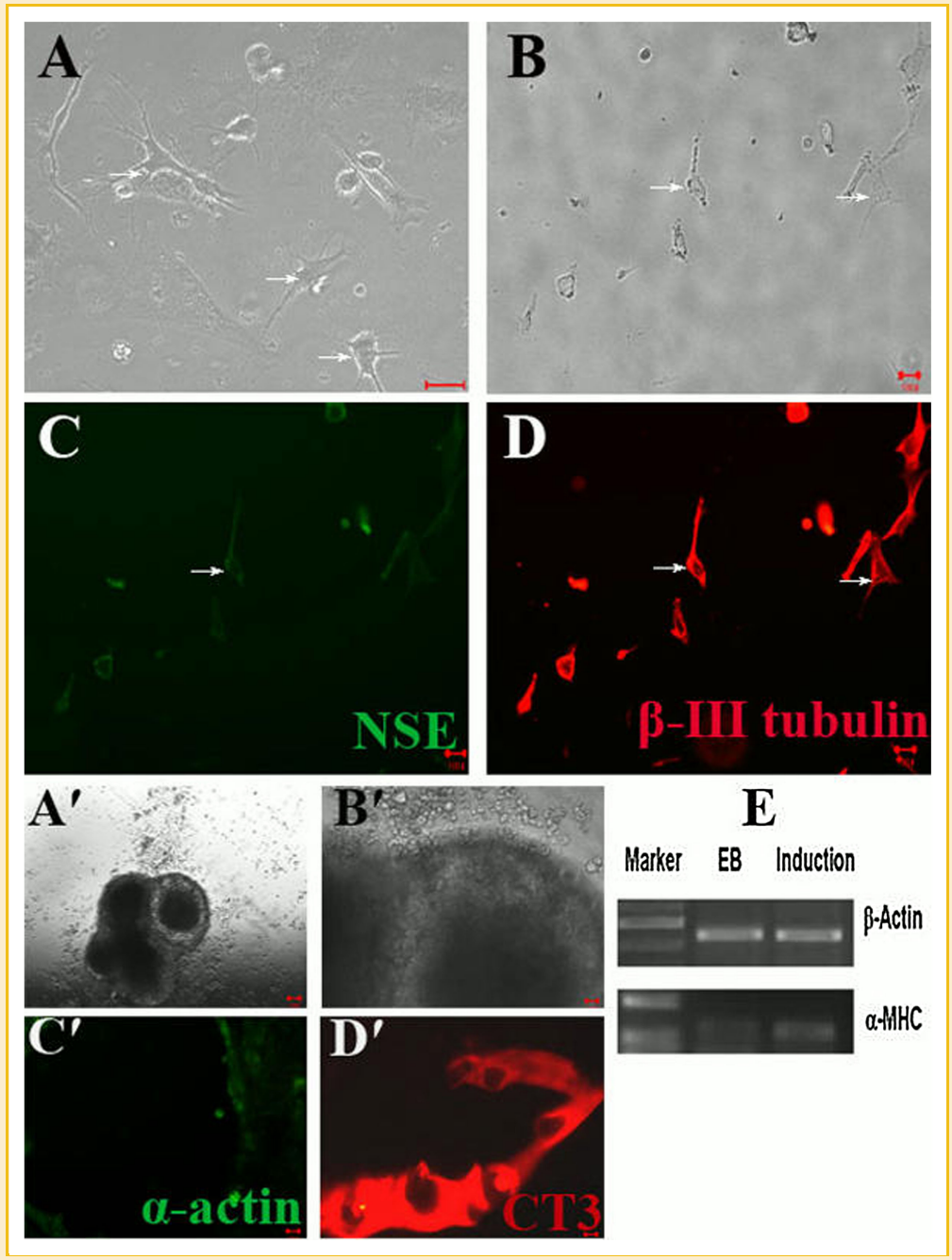

Fig. 6. Neural and cardiac differentiation by mGSCs induction. A,B: Neuron-like cells were observed after induction, bar = 50 μm (A), bar = 20 μm (B). C,D: NSE and β-III tubulin positive cells, bar = 20 μm (C,D). Heart beating clusters were formed in induced mGSCs EBs (A′,B′), bar = 50 μm (A′), bar = 20 μm (B′). Cardiac like cell clusters were positive for cardiac α-actin (C′) and CT3 (D′), bar = 20 μm (C′,D′): E′: The expression of cardiac specific marker α-MHC were up-regulated in cardiac like cell clusters analyzed by semi-quantitative RT-PCR.

After confirming the presence of mGSCs in the colonies, we induced GSCs to differentiate into spermatogenic cells by in vitro culture. EBs derived from mGSCs were cultured in 2×10^{-6} M RA in combination with testicular extracts (GE). After 1–3 weeks, extruded sperm-like cells and large round cells appeared (Fig. 7A,B). Light microscopy revealed a low number of presumptive spermatocytes and/or round spermatid-like cells defined according to their morphological properties [Tanaka et al., 2003]. Cells expressing Vasa (Fig. 7C), C-kit (Fig. 7D), FE-J1 (Fig. 7E), EMA-1 (Fig. 7F), and Scp3 (Fig. 7G,H) were observed in induced cultures. FE-J1 was specific for haploid male germ cells (mGCs). However, we could not histologically distinguish intercellular bridges. Dissociation of these structures revealed the presence of several cell types varying in size from 10 to 20 μm, and these cells were generally spherical with apparently intact plasma membranes. After 2 weeks of culture, Vasa, C-Kit, Scp3, β-integrin, EMA1, and FE-J1 genes were expressed in

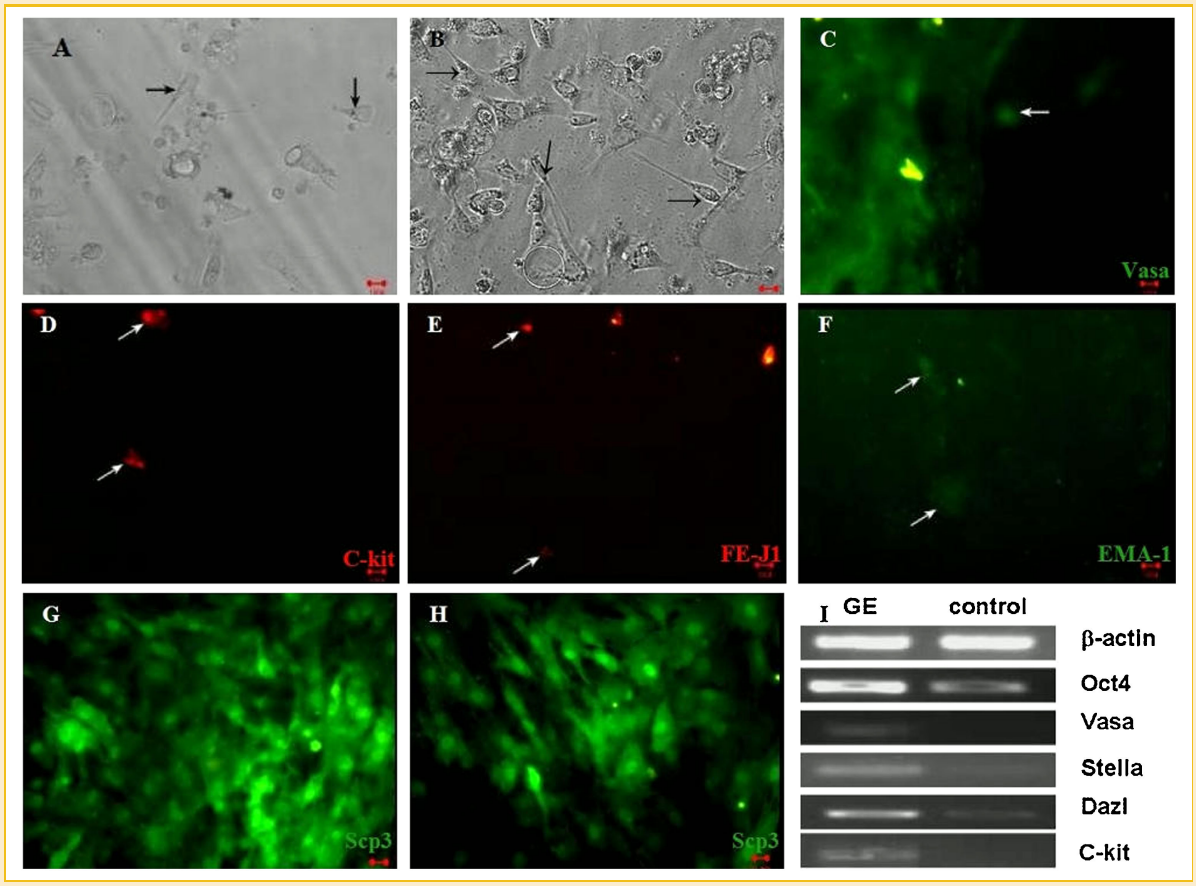

Fig. 7. Sperm-like cell differentiation derived from mGSCs by RA and testicular extracts induction. A,B: Sperm-like cells were appeared in induction; Induced cells were positive for Vasa (C), C-kit (D), FE-J1 (E), EMA-1 (F), and SCP3 positive cells were observed in induction (G,H). Bar = 20 μm. Expressions of germ cell markers including Oct4, Dazl, Stella, Vasa, and C-kit were up-regulated in GE induction (I).

induced cells, respectively, and some germ cell markers were up-regulated in RA combinated with GE medium, compared to control (Fig. 7I). These results demonstrate that our cultured GSCs are pluripotent with the capacity to generate many cell types including sperm-like cells [Ko et al., 2009].

DISCUSSION

RETINOL SUPPORTS SELF-RENEWAL OF mGSCs

Male GSCs maintain a balance between the production of mature sperm and self-renewal, thus being a valuable tool for studies on spermatogenesis, treating male infertility and genetically modifying the male germ line [Lee et al., 2006]. Recently, several groups reported to have obtained multi- or pluripotent stem cells from adult or neonatal mice testis [Izadyar et al., 2003; Kanatsu-Shinohara et al., 2004, 2008; Guan et al., 2006; Seandel et al., 2007; Izadyar et al., 2008; He et al., 2010]. Conrad et al. [2008] have developed a method for establishing human adult mGSCs from testicular biopsies. However, these studies all have some limitations, as samples were either obtained from neonatal mice or the detailed description of the potency and origin of the initial mGSCs was not provided. Ko et al. [2009, 2010] proved that germline-derived pluripotent stem (gPS) cells originated from unipotent adult GSCs and demonstrated that unipotent GSCs established from adult testis could be converted into pluripotent cells. Our study was consistent with Ko's report including the morphology, growth characteristics and potential differentiation of mGSCs derived from adult Kunming mouse testis [Ko et al., 2009, 2010].

Previous studies have shown that mGSCs proliferate when immortalized or co-cultured with feeders, followed by differentiation into spermatogenic cells by in vitro culture or transplantation [Feng et al., 2002; Kanatsu-Shinohara et al., 2003a]. In this study, we isolated and propagated mGSCs from testicular tissues of adult Kunming mice and they maintained an undifferentiated state in serum-free and feeder-free system. LIF, RE, GDNF can increase the efficiency of derivation of AP positive colonies, and these results were consistent with previous results [Kanatsu-Shinohara et al., 2003a; Kubota et al., 2004; Chen and Khillan, 2008]. Interestingly, we found that Clonal medium, a kind of commercial serum-free and feeder-free medium, which used to culture mouse ESCs, was beneficial for the derivation of mouse mGSCs, and our RE serum- and feeder-free culture system could maintain GSCs in an undifferentiated state. We also found that the pluripotent ES-like mGSCs could be successfully cultured in RE medium and RE up-regulated the expression of proliferation markers including C-myc and PCNA compared to KSR control. Moreover, several cell cycle

regulators, which could regulate the cell cycle progression during the G1/S transition, were also detected, therefore a better serum-free and feeder-free system may be found and extend the longevity of mGSCs. We chose to analyze the expression of cyclin D1 and cyclin A but not other cyclin proteins because cyclin D1 is expressed only in proliferating spermatogonia during spermatogenesis and plays a role in spermatogonial proliferation, in particular during the G1/S phase transition [Beumer et al., 2000], and cyclin A is a regulatory protein involved in control of the S phase of cell cycle [He et al., 2009]. We observed that both cyclin D1 and cyclin A were up-regulated by RE which indicated that both of them were involved in regulating cell cycle progression from G1 to S phase induced by RE. Simultaneously, these cells maintained the expression of pluripotent markers being similar to that of commercial serum-free and feeder-free medium: Clonal medium. Myc is activated through various mitogenic signals such as Wnt (wingless and int), Sonic hedgehog (Shh) and EGF (via the MAPK/ERK pathway). Myc is a very strong proto-oncogene and its overexpression stimulates gene amplification [Denis et al., 1991]. Myc activation results in numerous biological effects including driving cell proliferation (by up-regulating cyclins), and regulating cell growth (by up-regulating ribosomal RNA and proteins), apoptosis, differentiation, and stem cell self-renewal. PCNA is a distinct marker for the proliferative spermatogonia [Wrobel et al., 1996; Costoya et al., 2004], which was up-regulated in RE medium. These results demonstrated that RE may regulate C-myc, which results in the upward of PCNA and CDK2, cyclin A and cyclin D1 to maintain mGSCs self-renewal. Whether the true regulating mechanism of RE in mGSCs is similar to that in mGSCs, we need to further study PI3K/Akt or MEK/ERK pathway [Chen et al., 2007; Chen and Khillan, 2008; Chen and Khillan, 2010].

RETINOL MAINTAINS PLURIPOTENCY OF mGSCs

Previous reports claimed the generation of ES-like cells from human adult testis, but these ES-like cells had not fully acquired pluripotency, as determined by pluripotent gene expression and/or teratoma formation [Conrad et al., 2008; Kossack et al., 2009; Mizrak et al., 2009]. We also compared the gene expressions between ESCs and mGSCs analyzed by microarray, and the results showed that mGSCs were highly similar to pluripotent mouse ESCs, this is consistent with GSCs derived from human and mouse testis analyzed by RT-PCR, microarray and induction differentiation [Pesce M, Scholer HR. 2001; Kanatsu-Shinohara et al., 2004, 2008; Kubota et al., 2004; Seandel et al., 2007; Conrad et al., 2008; Ko et al., 2009, 2010; Kossack et al., 2009].

Conversion of unipotent PGCs into pluripotent embryonic germ (EG) cells was demonstrated in mouse and human models [Matsui et al., 1992; Shamblott et al., 1998]. This is consistent with our finding that unipotent GSCs can be converted into pluripotent cells under in vitro serum-free and feeder-free conditions. Commonly, somatic cells can only be converted into pluripotent cells upon introduction of a cocktail of exogenous transcription factors, named induced pluripotent stem (iPS) cells [Stadtfeld et al., 2008]. However, the systems were complex and the efficiencies were still low [Ko et al., 2009]. In this study, we demonstrated that mGSCs established from adult testis could be reprogrammed into pluripotent stem cells under relatively simple culture conditions. The observed differences between our study and previous reports may be due to a combination of factors, including medium composition, criteria used for identification of ESC-like cells, and a different microenvironment created within our culture system [Ko et al., 2009].

Previous studies have shown that unlike transplanted mGSCs, cells propagated and differentiated by in vitro cultivation usually displayed limited spermiogenesis [Rassoulzadegan et al., 1993; Hofmann et al., 1994; Hue et al., 1998; Lee et al., 2001; Feng et al., 2002]. Cells expressing FE-J1 specific for haploid mGCs, sperm-like cells in our induced cultures were obtained. Vasa, C-Kit, EMA1, Scp3 and FE-J1 genes were expressed in induced cells, respectively. Compared to control medium, some germ cell markers were up-regulated in RA medium combinated with testicular extracts. These results demonstrated that our cultured mGSCs were pluripotent with the capacity to generate many cell types including three germ layers and sperm-like cells. Dong et al. [2010] induced cattle mGCs into sperm-like cells by RA. To our knowledge, this is the first report describing the maintenance and differentiation of mGSCs from adult Kunming mouse testicular in RE serum-free and feeder-free medium. Use of these pluripotent male GSC lines derived from adult mouse testis may resolve current challenges with primary cultures of isolated spermatogonia and greatly increase the success and the efficiency in generating transgenic mice and research on cell differentiation. It will also be a useful model to analyze the mechanisms of infertility caused by genetic factors and drug toxicity.

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