

## ***In vitro* propagation of spermatogonial stem cells from KM mice**

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## **1. ABSTRACT**

Spermatogonial stem cells (SSCs) are a unique type of stem cells in that they transmit genetic information to the next generation by producing sperms. Studies of SSC proliferation and differentiation have been hampered by the inability of reconstructing these processes *in vitro*, particularly in a serum-free culture system. Several groups have reported the long term culture of SSCs during which SSCs self-renew and restore spermatogenesis when transplanted back to recipient testes. However, different protocols and mice with particular genetic background have been used by different laboratories, and the techniques have not been adopted widely. In the present study, we first established a SSC isolation and culture system composed of differential adherence selection of SSCs, serum-free medium and mouse embryonic fibroblast (MEF) feeder cells. SSCs from KM pups could be cultured on MEF feeders in StemPro-34 SFM Medium supplemented with glial cell line-derived neurotrophic factor (GDNF), soluble GDNF family receptor alpha-1 (GFRα1) and basic fibroblast growth factor (bFGF) for 1 month. These SSCs were characterized morphologically and by examining the expression of marker genes. Expression of Oct4 and Sox2, which are crucial factors in embryonic stem cell (ESC) self-renewal, were detected in our cultured SSCs, suggesting that SSCs may share with ESCs some common mechanisms in self-renewal regulation. We also found that LIF had no effect on the proliferation of cultured SSCs derived from KM mice.

## **2. INTRODUCTION**

Spermatogenesis in the testis is a very productive process by which a large number of male gametes are constantly produced from a small population of progenitor cells throughout post-pubertal life (1). These progenitor cells are termed spermatogonial stem cells (SSCs) and their origin could be traced back to the gonocytes of neonatal animals and even further to the primordial germ cells (PGCs) in early stage embryos (2). The start of spermatogenesis involves two related processes of SSCs--their self-renewal and differentiation initiation (3). The two processes are believed to be controlled by intracellular and extracellular factors. The extracellular factors are from SSC niche composed of Sertoli cells, extracellular matrix components, and other testicular cell types (4, 5). Apparently, *in vitro* culture, particularly serum-free culture of SSCs would be a very valuable means for the mechanism studies of SSC self-renewal and differentiation. This had long been difficult because of several problems. First, the number of SSCs in the testis is very low--there are only about  $2 \times 10^4$  SSCs out of  $10^8$  total cells in an adult mouse testis, that is, 1 in 5000 (1). Second, these cells could not be distinguished and separated from other type of testis cells. This is due to the fact that SSCs do not possess unique morphological features and molecular markers that could be used in cell sorting. Third, as a result of the above two problems, SSCs had long been just a function definition, and there had even been no functional assay even if the cells could be isolated and cultured *in vitro*.

Fortunately, such an assay, known as the germ cell transplantation technique, was established by Brinster *et al* in 1994 (6), marking a milestone in the journey of spermatogenesis study.

As a matter of fact, *in vitro* culture of germ cells has been attempted by different laboratories without the use of a functional assay. For long-term culture, testicular cells were transfected by using SV40 large T antigen (7) or immortalized with telomerase reverse transcriptase gene (8). Proliferation and differentiation of germ cells have been observed. However, without functional confirmation, whether the cultured germ cells were really SSCs is questionable. The first convincing *in vitro* culture of SSCs was again reported by Brinster's group in 1998 (9). In their work, SSCs could be cultured for approximately 4 months, and reestablished spermatogenesis following transplantation back to recipient testes. The mouse strain used was B6.SJL, the culture medium was the Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal calf serum (FCS), and SIM mouse embryo-derived thioguanine and ouabain resistant (STO) feeder cells were required. SSCs were either from neonates or adult mice. Unfortunately, further work using this system and ROSA26-B6/129 mice indicated that the number of SSCs decreased in a time-dependent manner, and only 24% stem cells remained by the 7<sup>th</sup> day in culture (10). However, several important observations were made: First, some feeder cell types (OP9 bone marrow stroma and L fibroblast cell lines) were better than STO in SSC maintenance while some (TM4 or SF7 Sertoli cell lines) were worse; Second, some growth factors (activin A and bone morphogenetic protein 4, BMP4) reduced the maintenance of SSCs while others such as GDNF promoted it; Third, medium MEM $\alpha$  was better than DMEM in SSC maintenance and extra supplements seemed not necessary. These observations eventually led to the establishment of a serum-free long-term culture system of SSCs that consisted of enriched SSCs in culture initiation, MEM $\alpha$ , mitotically inactivated STO feeders, and some growth factors (11, 12). In this system, SSCs were enriched by Fluorescence-Activated Cell Sorting (FACS) or Magnetic-Activated Cell Sorting (MACS) with anti-Thy-1 antibody. SSCs from DBA/2J  $\times$  ROSA mice only required GDNF for *in vitro* expansion, cells from C57  $\times$  ROSA required one additional protein factor, bFGF or soluble GDNF-family receptor  $\alpha$ 1 (GFR $\alpha$ 1), and cells from three inbred strains, 129\_SvCP, C57BL\_6, and SJL, could be expanded *in vitro* when both factors were added. They also found that this system supported the expansion of SSCs from neonates, pups, as well as cryptorchid or wild-type adult mice. It seemed that addition of serum to the medium at least inhibited the expansion of SSCs of C57  $\times$  ROSA mice.

A different culture system was developed by Shinohara's group in Japan (13). Several differences were noticed. First, testicular cells were isolated from neonates of the DBA/2 background mice. Second, the system used a very rich Medium StemPro-34 SFM supplemented with StemPro supplement and a lot of other hormones, nutrients and protein factors such as  $\beta$ -estradiol, progesterone, mouse epidermal growth factor (EGF), human basic fibroblast

growth factor (bFGF), murine leukemia inhibitory factor (LIF), rat GDNF, as well as 1% FCS. Third, stem cells were enriched by separating germ cells and somatic cells in culture plate because somatic cells adhere to the gelatin-coated plate more quickly. In addition, MEF instead of STO feeder cells were used. The authors termed their stem cells germline stem cells (GSCs) instead of SSCs since they were from gonocytes of neonates. Later the same group claimed that the low concentration of serum (0.3 to 2%) was required for the establishment of GSCs but not their expansion (14). Once the colonies of GSCs were formed, the cells could expand on MEF feeders in serum-free medium that was supplemented with B27, a proprietary serum-free supplement designed for long-term culture of neurons. Or the GSCs were able to expand just on laminin-coated plate with 1% FCS in the medium. By using a very similar procedure, it was reported that SSCs from adult DBA/2 mice could also be cultured successfully (15).

As can be seen, the two SSC culture systems described above differ in several aspects. The major difference is that the somatic cells were removed quickly by FACS or MACS in the first system while, in the second one, their removal is much slower by differential adherence selection. While serum in SSC culture medium is completely eliminated from the beginning with the first system, low concentration of serum is included at least in the establishment stage of GSC colonies with the second system. The authors of the first system succeeded in SSC culture from several mouse strains while those of the second system only performed SSC cultures derived from DBA/2 mice. Based on these observations, we thought the second system was more feasible under our research conditions, and some modifications/improvements could be made. We would also like to test whether SSCs from a different mouse stock, the KM mice, could be cultured. In our procedures, we included 10% FCS in the culture medium in the initiation stage of SSC culture in order to remove the somatic cells in a shorter time span. The serum was completely removed when SSCs were maintained on MEF feeder cells. By using this modified system, we successfully cultured SSCs from KM pups for one month. Expression of marker genes especially those that could play a role in supporting SSC proliferation was examined by both immunocytochemistry and RT-PCR. Our results indicated that Oct4 and Sox2 proteins, essential transcriptional regulators in ESC self-renewal, could also be involved in the maintenance of SSC *in vitro* proliferation while LIF is not required in this process.

## 3. MATERIALS AND METHODS

### 3.1. Experimental animals

Three mouse lines ICR, DBA/2 and KM were all obtained from Beijing Weitong River Laboratory Animal Inc., China. Animals were housed under 16-h light, 8-h dark schedule with food and water ad libitum, and treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All the protocols were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

### 3.2. Culture Conditions

The medium for the differential adherence selection of testis cells was DMEM/F12 (Sigma-Aldrich) supplemented with 2 mM L-glutamine (Invitrogen Corp.), 10% FCS, 100 unit/ml penicillin and 100 µg/ml Streptomycin (Invitrogen Corp.). This medium is referred to as selection medium (Medium D). The culture medium (Medium S) for SSCs was consisted of StemPro-34 SFM (Invitrogen Corp.) supplemented with StemPro nutrient supplement (Invitrogen Corp.), 25 µg/ml insulin, 100 µg/ml transferrin, 60 µM putrescine, 30 nM sodium selenite, 6 mg/ml D-(+)-glucose, 30 µg/ml pyruvic acid, 1 µl/ml DL-lactic acid, 50µM 2-mercaptoethanol, 100nM ascorbic acid, 10 µg/ml d-biotin (Sigma-Aldrich), 5 mg/ml bovine albumin (MP biochemicals, formerly ICN), 2 mM L-glutamine, minimal essential medium (MEM) vitamin solution (Invitrogen Corp.), MEM nonessential amino acid solution (Invitrogen Corp.), 10 ng/ml recombinant human bFGF (BD Biosciences, Inc.), 20 ng/ml recombinant rat GDNF (R&D Systems, Inc.), 200ng/ml recombinant rat GDNFsRα1(GFRα1)/Fc chimera (BD Biosciences, Inc.). MEF cells were cultured in DMEM supplemented with 10% FCS, 50 µM 2-mercaptoethanol, 2 mM L-glutamine and antibiotics. For preparing feeder layers, MEF cells were treated with 10 µg/ml mitomycin C (Sigma-Aldrich) for 2-2.5 hours and then plated at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> to wells pre-coated with 0.2% gelatin (Sigma-Aldrich) in the same medium. All cells were maintained at 37°C in an atmosphere of 5% carbon dioxide in air.

### 3.3. Isolation and collection of mouse testis cells

Testis cells from 4-5 days postpartum (dpp; day of birth is designated as 0 dpp) pup males were collected by a two-step enzymatic digestion method previously described (16) with some modifications. Briefly, testes were first decapsulated and treated with 10 volumes of Digestion solution I (2mg/ml collagenase type IV and 200µg/ml DNase (Sigma-Aldrich) in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS) at room temperature for 3-5 minutes with gentle agitation until the tubules were separated. Dispersed testis tubules were then washed 3 times in 10 volumes of PBS by centrifugation at 170g for 2 minutes each time. For the second digestion, collected specimens were treated with 5 volumes of Digestion solution II (2mg/ml collagenase type IV, 200µg/ml DNase and 2mg/ml hyaluronidase (Calbiochem) in serum-free DMEM (Invitrogen corp.) at room temperature for 2-5 minutes with vigorous aspiration until clumps of tubules were no more visible. Digestion was then stopped by addition of 5ml PBS or DMEM medium with 10% FCS. Cells were collected by centrifugation at 200g for 5 Minutes, resuspended in PBS and then filtered through a nylon mesh with 60µm pore size to remove large clumps of cells. The filtrate was washed 2 times in PBS by centrifugation at 200g for 5 minutes each time. The cells were finally resuspended in Medium D and plated on 0.2% (w/v) gelatin-coated tissue culture flasks (25cm<sup>2</sup>) or wells of 6-well (9.4cm<sup>2</sup>/well) or 12-well (3.83cm<sup>2</sup>/well) tissue culture plates at a density of  $2 \times 10^5$  cells/cm<sup>2</sup>. Approximately  $4 \times 10^5$  cells were collected from one pup testis by this procedure. The number of dead cells was generally less than 2% as assessed by trypan blue staining.

### 3.4. Differential adherence selection and culture procedures of SSCs

Dissociated testis cells on the gelatin-coated plates were first incubated in Medium D for 1 hour. After gentle pipetting, the floating cells were recovered and transferred to a second plate, and then incubated for 3 to 4 hours. Following vigorous aspiration, suspended cells were transferred to a third plate and cultured overnight or for 1 day until the remaining somatic cells fully attached to the bottom of plates and began to grow. Germ cells were then collected by pipetting and transferred to a fourth plate with mitomycin C-inactivated MEF feeder layer and Medium D was replaced by Medium S at this time. Usually about 3 - 4 days after culture initiation, colonies of SSCs could be observed. In order to further eliminate somatic cells, instead of regular passage which uses trypsinization, only the floating germ cells were transferred to new plates with MEF feeders by using the pipetting method. After another 2 to 3 passages with MEF feeder cells, SSCs started to expand steadily. Thereafter, established SSCs with MEF feeders were dispersed by 0.2% collagenase treatment, replated to new plates with MEF feeders, and passaged every 2-4 days at a one-third to one-fourth dilution depending on their proliferation state. The medium was partially changed for one-third every day.

### 3.5. RT-PCR analysis of marker gene expression

Total RNA was extracted using Trizol reagent (Invitrogen Corp.). For reverse transcription polymerase chain reaction (RT-PCR), first-strand cDNAs were synthesized with Superscript<sup>TM</sup> III Reverse Transcriptase (Invitrogen Corp.), and PCR was carried out with recombinant Taq DNA Polymerase (Takara) according to the manufacture's instruction. All primer sequences were listed in Table 1. G3PDH was used as a normalization control. PCR products were electrophoresed on 1.0% agarose gels.

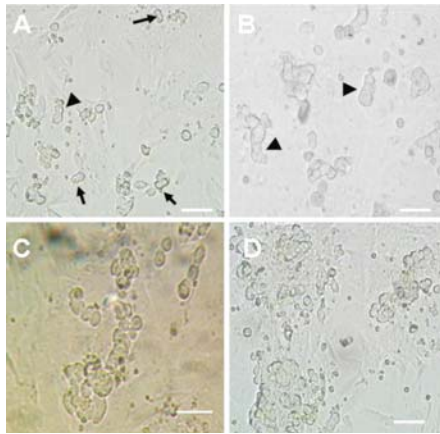
### 3.6. Immunocytochemical staining and confocal laser microscopic observation of SSCs

The primary antibodies used in this study were biotin-conjugated rat anti-CD9 monoclonal antigen (BD Biosciences), rat anti-mouse CD117 (c-kit) biotin-conjugated monoclonal antibody (Chemicon), rabbit anti EP-CAM (H-70) polyclonal antibody (Santa cruz biotechnology, Inc.), mouse anti-human c-Ret polyclonal antibody (R & D systems), mouse anti-human CD56 (NCAM) monoclonal antibody (BD Biosciences), rabbit anti-Oct4 affinity purified polyclonal antibody (Chemicon), rabbit anti-GFRα1 polyclonal antibody (Chemicon) and R-PE-conjugated rat anti-mouse CD90.2 (Thy-1.2) monoclonal antibody (BD Biosciences). The secondary antibodies included FITC-conjugated goat anti-rat IgG, PE-conjugated donkey anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG (Santa cruz biotechnology, Inc.), TRITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich) and TRITC-conjugated streptavidin (Invitrogen Corp.).

Immunocytochemical staining was performed according to the standard procedure. Briefly, cultured cell clumps in wells of a 4-well dish (Nunc) with MEF

**Table 1.** List of mouse primers used

Name	Forward primer (5' → 3')	Reverse primer (5' → 3')	Size of band (bp)
Oct4	GGCGTTCTCTTTGGAAAGGTGTTTC	CTCGAACACATCCTTCTCT	313
Sox2	GGCAGCTACAGCATGATGCAGGAGC	CTGGTCATGGAGTTGTACTGCAGG	130
Nanog	CTATGATCTTTCTTTCTAGACACTGAG	GACAATTAGAGCTATGCAGAGAAACC	1332
Mvh	GCTCAAACAGGGTCTGGGAAG	GGTTGATCAGTTCTCGAG	130
Dazl	GCCAGCACTCAGTCTTCATC	GTTGGAGGCTGCATGTAAGT	418
Fragilis	TGCCTTTGTCCGCACCAT	GGGTGAAGCACTTCAGGACC	468
Piwi2	CCGTCATGAAGGAGAGCTCG	GGAACGACTCTGTGCTGGAT	347
Pum1	TGGAGGCGGCTATAATACTA	TTCTTTTCACCTTTGTCGT	471
Pum2	TAGAATCTCGGGGAATGGGA	TGGTTTATTTCGCTGTCGGAT	602
c-kit	TGCTCTGCGTCTCTGTTGGT	CGTCGCTGTCTTCTTTGCC	385
Zfp145	TGCTGCCCAACCACAAGG	CGCTGAATGAGCCAGTAAAT	362
c-Ret	CTGCCGCTGCTAGGAGAAGCCCCAC	CTTCACACTGATGTTGGGACAAAGGAA	555
ngn3	AAATCCCTCTGGGTCTCATCACTGC	CGCCGAGTTGAGGTTGTGC	534
GFR $\alpha$ 1	ACTCTGGATTGTGATGTCCG	CGCTGCGCACTCATCCTT	191



**Figure 1.** SSC colonies from KM pup mice on day 1 (A), 3 (B), 7 (C) and 28 (D) after being plated on MEF feeder cells. SSCs with the  $A_{paired}$  (arrow) and  $A_{aligned}$  (arrow head) spermatogonia morphology could be seen. Scale bar: 50  $\mu$ m.

feeders were washed 2 times in PBS and fixated with 4% paraformaldehyde for 20 minutes at room temperature. After washed twice in PBS, cells were blocked in PBS containing 1% BSA, 0.1% Triton X-100 and 2% normal goat serum at room temperature for 45 minutes. The samples were then incubated overnight at 4°C with primary antibody diluted as described by manufacturers. After washed 3 times with PBS containing 1% BSA, the cells were incubated with diluted secondary antibody for 1 hour at room temperature in the dark followed by 3 times of wash, and covered with 0.5 ml PBS. Images were acquired using Leica confocal microscope. For negative controls, the cells were incubated with non-immune rabbit or mouse serum, followed by incubation with the second antibodies.

### 3.7. Flow cytometric analysis (FCA) of cells

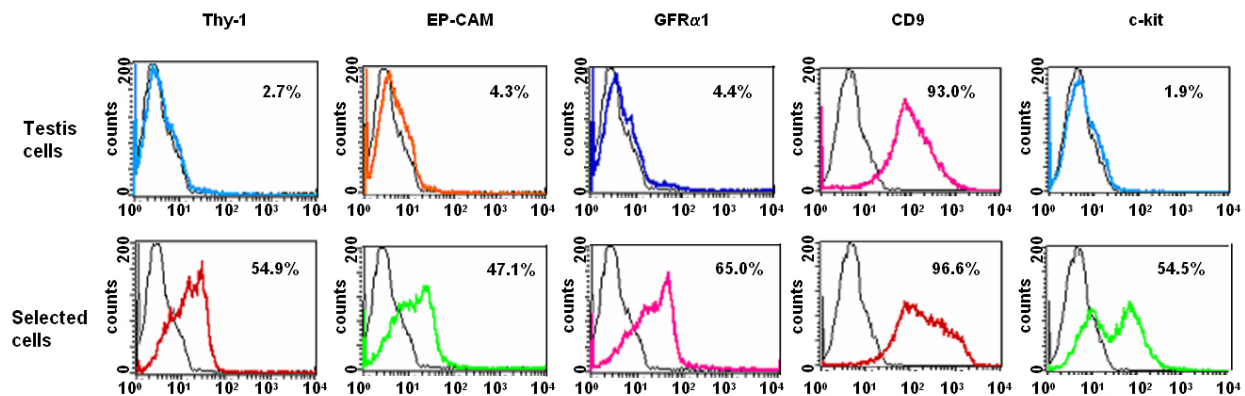
Cells in single cell suspension were washed twice in PBS containing 10% newborn calf serum (NCS) and collected by centrifugation at 250g for 5 minutes. At the final step, the pellet was gently resuspended with 50  $\mu$ l cold analysis buffer (2% NCS and 0.1% sodium azide in  $Ca^{2+}$ - and  $Mg^{2+}$ -free PBS) for a final concentration of  $1 \times 10^7$  cells/ml. PE-conjugated antibodies were added and the dilutions were according to manufacturer's instructions. The tube was vortexed briefly and incubated for 30 minutes at 4°C in the dark. The cells were

then washed twice with 1ml analysis buffer and finally resuspended in 1 ml buffer and incubated at 4°C (or on ice). FCA was then conducted on the FACS-Calibur system (BD Biosciences), and 10,000 events were collected. For indirect staining, unlabelled primary antibodies and FITC-conjugated secondary antibodies were used and the dilution was according to the manufacturer's instructions.

## 4. RESULTS

### 4.1. An improved procedure for SSC isolation and culture

Although several groups have reported their successes in SSC isolation and culture, we have noticed that no two laboratories have used exactly the same protocol, implying it is still a tricky task for others to repeat one's result exactly (11-13). In the present study, we have developed a modified procedure for SSC isolation and culture based on the previously reported one (13, 15). Testis cells were isolated by using the two-step enzymatic digestion method (16). However, we performed the digestion at room temperature (22-25°C). Importantly, in the second digestion collagenase instead of trypsin were used, and seminiferous tubules were agitated by vigorous pipetting during the digestion to reduce the total digestion time to within 10 minutes. These modifications helped to keep the viability of testis cells. During the initiation stage of testis cell culture, somatic cells but not SSCs adhered to the gelatin-coated plate quickly. As a result, SSCs could be enriched by collecting the floating cells and transferring them to new plates sequentially. We called this process differential adherence selection. The medium used during this stage was named as Medium D which contained 10% FCS. Culture with Medium D enabled the enrichment of SSCs much quicker as compared with the time spans reported by others (13, 15). It took only 40-60 minutes for somatic cells to adhere to the bottom of the first plate. When floating cells were transferred to the second plate, the attachment needed 3 to 4 hours. After overnight- to 1 day culture in the third plate, Medium D was replaced by Medium S, and highly enriched SSCs could be transferred to the fourth plate with mitomycin-inactivated MEF feeder cells after vigorous pipetting. Using these procedures, very few germ cells were left on the original gelatin-coated plate, and transferred cells were highly germ cell enriched. Usually about 3- to 4 days from the beginning, the transferred germ cells cultured in Medium S with MEF feeders proliferated and formed colonies (Figure 1 A and B).



**Figure 2.** Flow cytometric analysis of testis and selected cells positive for surface markers. Cells collected by vigorous pipetting at the end of overnight culture were incubated with antibodies against thy-1, EP-CAM, GFR $\alpha$ 1, CD9 and c-kit. Black lines, control immunoglobulin; color lines, specific antibodies.

#### 4.2. Differential adherence selection for male germ cells is a simple but effective approach

Cell surface protein molecules Thy-1 (17), EP-CAM (18), GFR $\alpha$ 1 (12), and CD9 (19) have been found to be expressed in SSCs, and could be used in FCA to check the efficiency of SSC enrichment by our differential adherence selection method. After vigorous agitation, the floating cells from the third plate were trypsinized, collected by centrifugation and then incubated with antibodies against these molecules respectively. FCA results in Figure 2 indicated that the numbers of Thy-1, EP-CAM, GFR $\alpha$ 1 positive SSCs were increased by 20.3, 11, 14.8 folds respectively. 93.0% of the testis cells were CD9 positive, and the positive cells were enriched only by 1.04 folds. Clearly, the enrichment of SSCs can not be accessed by the fold change of CD9 positive cells as it has been known that interstitial cells are also positive for CD9 (19).

This observation was consistent with the data in a previous study where 83% testis cells were CD9 positive (20). We also examined the numbers of the cells positive for c-kit, a marker of differentiated spermatogonia (21) before and after the selection. It seemed that the differentiated spermatogonia were also enriched by about 28 folds. This was reasonable as not all of the cells at this stage could form SSC colonies in the later stage of culture. Differentiated germ cells were eliminated in prolonged time of culture. These results suggested that the differential adherence selection is very effective in comparison with enrichment by MACS where SSCs could be enriched by 6-, 30-, 4-, and 5-fold for cryptorchid adult and wild-type adult, pup, and neonate, respectively as accessed by transplantation assays (11).

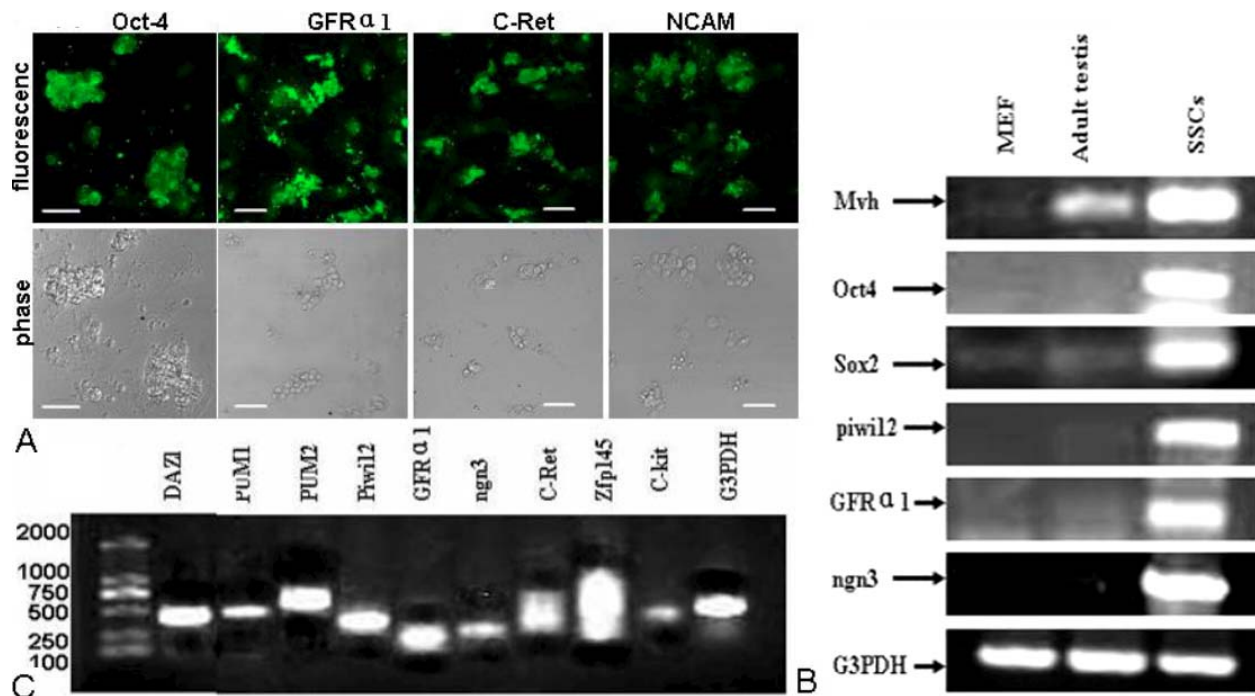
#### 4.3. Culture and phenotypic characterization of SSCs from KM pup mice

SSCs from mice of DBA/2 background and C57BL/6, SJL, and 129/Sv inbred mice could be cultured for a long time by using the protocol from Brinster's group (11, 12). However, only SSCs from DBA/2 mice could be long-term cultured by using the protocol from Shinohara's group (13, 14). Our protocol was derived

from the modification of the Shinohara's protocol. By using our protocol, SSCs from DBA/2 and ICR mice could be cultured for about one month (data not shown). KM mice are outbred stock originated from Swiss mice when they were introduced from Hoffkine Institute of India to Kunming, China in 1994, and widely used by scientists in China. SSCs from KM mice could also be cultured for about one month with 6 passages (Figure 1). SSC colonies appeared on MEF feeder layers 3 to 4 days after culture initiation, and could also be observed on testis somatic cell layers if cells were let stay in the third plate for a longer period. Generally, by day 10 to 12 after culture initiation, SSC cultures were steadily established. The *in vitro* doubling time of KM SSCs was 48 to 72 hours. SSCs from KM pups in culture showed typical morphological features (Figure 1). They formed colonies of 4-50 cells. Some colonies had the shape of paired (Figure 1 A, arrows) or aligned (Figure 1 A and B, arrow heads) undifferentiated spermatogonia connected by cytoplasmic bridges. More frequently colonies appeared as a large cell mass with an irregular contour (Figure 1 C and D). These results were reproducible, and similar cultures were established from more than 40 respective experiments.

To confirm the cultured cell clumps are SSC colonies, we checked the protein or RNA expression of a panel of SSC marker genes. We first examined the protein expression of GDNF receptor components GFR $\alpha$ 1, c-Ret and NCAM with immunocytochemistry (Figure 3 A). GFR $\alpha$ 1, c-Ret and NCAM are all subunits of GDNF receptor and have been known to be expressed in undifferentiated spermatogonia and cultured SSCs (12, 22, 23). All of markers were positive on the cell colonies as shown in Figure 3 A. The RNA expression of germ cell marker genes that are expected to be expressed in SSCs were examined by RT-PCR. As expected germ line marker genes, Mvh (24), a gamete marker gene, and Dazl (25), a germ cell-specific gene expressed premeiotically were all detected by RT-PCR. Pum1 and Pum2 are two RNA binding proteins. While Pum1 is expressed in multiple





**Figure 3.** Protein and RNA expression of marker genes in cultured SSCs from KM pup mice detected by immunocytochemistry (A) and RT-PCR (B and C). A) Oct4, GFRα1, c-Ret and NCAM expression detected by immunocytochemistry. Note that all cells in germ cell clumps are stained positive for these markers. Scale bar: 50μm. B) Semi-quantitative RT-PCR analysis of marker gene expression in SSCs in comparison with MEF and adult testes. C) The expression profile of marker genes in cultured cells.

tissues, Pum2 is most abundant in the testis and ovary (26). RNA expression of both genes was also detected in our cultured SSCs. These results indicated that our cultures contained germ cells. In order to further examine the cultured cells possess the properties of SSCs, we detected the expression of SSC marker genes. piwil2, known also as Mili that has been shown to play an important role in the maintenance of SSCs (27), Neurogenin3 (ngn3) (28) and zfp145 (29), two genes that are only expressed in undifferentiated spermatogonia, were all detected in our cultures. Expression of GFRα1 and c-Ret was also confirmed by RT-PCR. We also checked the expression of c-kit, a differentiated spermatogonia marker (21). Low level RNA expression of c-kit was detected by RT-PCR (Figure 3 C) but not by immunocytochemistry (data not shown), indicating that a small number of differentiated spermatogonia existed in our cultures. As a result, the data indicated that the cultured cell clumps mainly consisted of SSCs.

#### 4.4. Presence of Oct4-Sox2 regulatory complex in SSCs

Two transcription factors Oct4 and Sox2 play essential roles in pluripotency maintenance of ESCs. Oct4, a homeodomain transcription factor, has been well known for its expression in pluripotent cells of the preimplantation embryo, PGCs, undifferentiated spermatogonia as well as in oocytes (29-31). Sox2, a HMG-domain DNA binding protein, is expressed in both ESCs, neural stem cells (NSCs), and plays an indispensable

role in primitive ectoderm formation (32). As shown in Figure 3, the two factors were detected in our cultured SSCs by RT-PCR. The protein expression of Oct4 was also confirmed by immunocytochemistry. However, the expression of Nanog, an another homeodomain transcription factor essential for ES cell self-renewal maintenance, was not detected in our cultured SSCs (data not shown), which is consistent with previous reports that Nanog was expressed in compacted morula, ICM, epiblasts, and PGCs before colonization but not in adult testis and GS cell lines (33-35). Oct4 and Sox2 in the form of heterodimer affects the expression of several genes including Oct4 itself in mouse ES cells (36-40). It has recently been shown that Oct4, Sox2 and Nanog function as key players by co-occupying a substantial number of their target genes and are believed to form the core transcriptional regulatory circuitry in ESCs (41). Our results indicated that the Oct4 and Sox2 but not Nanog might play an important role in the self-renewal of SSCs via activating the expression of target genes.

#### 4.5. LIF is not required for *in vitro* proliferation of SSCs

LIF has been utilized to maintain the *in vitro* self-renewal of mouse ES (42, 43) and EG (44-46) cells. However, LIF is not required for self-renewal of human ES cells and several mouse ES cell lines (47-49). Moreover, LIF knockout mice develop normally (50). Although previous studies indicate that LIF/gp130 signaling do not greatly affect the activity of SSCs *in vitro* (12, 51), we

would like to confirm that it is also true on SSCs from KM mice. We added 250, 500 and 1000 units/ml of LIF to the Medium S respectively, none of three concentrations showed any difference with the control without LIF in terms of the time required to establish SSC cultures and the morphology of the colonies. These results indicated that LIF is not required for *in vitro* proliferation of SSCs.

## 5. DISCUSSIONS

Previous reports have shown that SSCs can be derived from neonatal and adult mice by adherence selection (13, 15). However, the time used to establish SSC cultures in these studies were much longer ranging from 15 to 25 days. In present study, the stable establishment of SSC cultures only needed 6- to 8 days. Several factors might contribute to this improvement. First, cultured SSCs from neonatal mice (0 to 2 dpp) were originated from gonocytes, which might need longer time *in vitro* to finish the transition from gonocytes to SSCs. In contrast, our cultured SSCs were from 4 to 5 day old pup males. These cells have arrived at the basement membrane of seminiferous tubules and completed the transition from gonocytes to SSCs, and are more active in mitosis. Second, the removal of somatic cells by our protocol was quicker, which required 2-4 days. Digestion using collagenase and addition of 10% serum to Medium D both helped somatic cells to adhere to the bottom of culture dishes quickly. Third, digestion accompanied by agitation at room temperature dramatically reduced the digestion time and enhanced the vitality of digested testis cells.

Previous reports indicated that mice of different genetic background required different culture conditions (12, 13). One group has only used mice of DBA/2 background with their culture system (13). The other group used mice of several backgrounds with their system. While SSCs of the DBA/2 mice only needed GDNF in the serum free medium to proliferate, the other strains required extra factors (12, 13). By using our culture protocol, SSCs from ICR, DBA/2 could be cultured for about 1 month. Importantly, we reported for the first time that the SSC cultures derived from KM mice could be easily established. Because our protocol was modified from Shinohara's by which only DBA/2 mice could be used for SSC culture, we believe the culture system based on differential adherence selection plus StemPro serum-free medium could be extended to mice other than just DBA/2. We failed to culture KM SSCs with our culture medium supplemented with GDNF only. Therefore, proliferation maintenance of KM SSCs seemed to require at least the activation of both GDNF and bFGF signaling pathways.

The GDNF signaling pathway has been shown to be critical for the cell fate decision of SSCs (22, 52, 53). GDNF is secreted by sertoli cells and signals through the receptor GFR $\alpha$ 1, which activate the receptor tyrosine kinase c-Ret (11, 53). NCAM together with GFR $\alpha$ 1 form a second GDNF receptor complex leading to a different signal transduction cascade (54). The GDNF signaling transduction has been reported to activate PLZF (promyelocytic leukemia-associated protein), a zinc finger

transcription factor, which is involved in the self-renewal of SSCs (29). All these signaling pathway components were detected in our cultured SSCs, indicating the importance of this pathway in the maintenance of SSC *in vitro* proliferation.

Oct4 is expressed by all pluripotent cells during mouse embryonic development, and it is also present in ES, EC (embryonic carcinoma), EG (embryonic germ) cell lines (55-58), and SSCs in adult mice (29). Oct4 usually interact with other transcription factors to activate or repress gene expression in mouse ES cells. One known partner is Sox2 that is expressed in both ESCs and neural stem cells (NSCs) (32), and whose inactivation results in defective primitive ectoderm (32). It has been known that the heterodimer of Oct4 and Sox2 affects the expression of several genes in mouse ES cells (36-40). The coexistence of Oct4 and Sox2 in cultured SSCs proposes that the Oct4-Sox2 complex also functions in SSCs.

It has been shown that the activation of LIF/gp130 signaling pathway is essential and sufficient for self-renewal of mouse ES cells (46), and is required for survival and proliferation of PGCs and EGCs *in vitro* (59). Although gp130 expression was detected on proliferating SSCs (12), the LIF/gp130 pathway seems to be dispensable for the derivation and maintenance of SSCs. This notion was again confirmed by our results as none of three concentrations of LIF used showed any improvements in SSC culture.

In summary, for the first time we have shown that SSC cultures could be established from KM mice by using a modified system composed of differential adherence selection, serum-free defined medium and MEF feeders. We propose that Oct4 and Sox2 may participate in the proliferation regulation of SSCs. We also indicated that LIF was not required for the self-renewal of SSCs *in vitro*.

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**Key Words:** Spermatogonial Stem Cell, Proliferation, Serum-Free Culture, Glial Cell Line-Derived Neurotrophic Factor, long-term culture

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