Zygote **22** (August), pp. 395–403. © Cambridge University Press 2013
doi:10.1017/S0967199413000142 First Published Online 14 May 2013

Effects of testicular interstitial fluid on the proliferation of the mouse spermatogonial stem cells *in vitro*

Peng Wang[2], *Yi Zheng*[2], *Ying Li*[2], *Hua Shang*[2], *Guang-Xuan Li*[2], *Jian-Hong Hu*[1] *and Qing-Wang Li*[1]
College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, P.R. China

Date submitted: 29.07.2012. Date accepted: 25.09.2012

Summary

Spermatogenesis is a process in adult male mammals supported by spermatogonial stem cells (SSCs). The cultivation of SSCs has potential value, for example for the treatment of male infertility or spermatogonial transplantation. Testicular interstitial fluid was added to culture medium to a final concentration of 5, 10, 20, 30 or 40%, in order to investigate its effects on proliferation of mouse SSCs *in vitro*, Alkaline phosphatase (AKP) assay, reverse transcription polymerase chain reaction (RT-PCR) analysis and indirect immunofluorescence of cells were performed to identify SSCs, and the proliferation rate and diameters of the SSCs colonies were measured. The results showed that the optimal addition of testicular interstitial fluid to culture medium was 30%. When medium supplemented with 30% testicular interstitial fluid was used to culture mouse SSCs, the optimum proliferation rate and diameter of the cell colonies were 72.53% and 249 μm, respectively, after 8 days in culture, values that were significant higher than those found for other groups ($P < 0.05$). In conclusion, proliferation of mouse SSCs could be promoted significantly by supplementation of the culture medium with 30% testicular interstitial fluid. More research is needed to evaluate and understand the precise physiological role of testicular interstitial fluid during cultivation of SSCs.

Keywords: Mouse, Proliferation, Spermatogonial stem cells, Testicular interstitial fluid

Introduction

Spermatogenesis is a complex and male-specific process supported by spermatogonial stem cells (SSCs), the only cells that contribute genes to subsequent generations (Huckins, 1971; Clermont, 1972). Testis cells are made up of somatic cells and reproductive cells. In the mouse, it is estimated that there are 2×10^4 stem cells in the total 10^8 cells, approximately, in the testis (Brinster & Nagano, 1998). Furthermore, a large percentage of germ cells are mitotic spermatogonia, spermatocytes or highly differentiated terminal stages cells (Uchida *et al.*, 1993; Morrison *et al.*, 1995). There is enormous value in understanding the biological characteristics of SSCs through the study of maintenance and proliferation of SSCs *in vitro*. At this time, many studies are focusing on understanding cultivation, self-renewal and differentiation of SSCs (Nagano *et al.*, 1998; Lee *et al.*, 2003; Kubota *et al.*, 2004; Kanatsu-Shinohara *et al.*, 2007; Oatley *et al.*, 2007; Araki *et al.*, 2010; Nolte *et al.*, 2010).

Stem cells are considered to grow in a special environmental niche supplemented with various factors that contribute to regulating proliferation and differentiation (Spradling *et al.*, 2001). Although the essential roles of growth factors have been explored, little information is known about the exact mechanism of proliferation or differentiation of SSCs. Analysis of SSCs directly *in vivo* has proved to be very difficult, as the SSC number in a testis is too small for easy study (Tegelenbosch & Derooij, 1993). To overcome this problem, some *in vitro* culture systems for SSCs have been developed (Kanatsu-Shinohara *et al.*, 2003). It has been demonstrated that glial cell line-derived neurotrophic factor (GDNF) is beneficial to maintaining SSCs *in vitro* (Tegelenbosch & Derooij, 1993; Nagano *et al.*, 2003). A complex and undefined medium was shown to be beneficial for

[1]All correspondence to: Jian-Hong Hu or Qing-Wang Li. College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, P.R. China. Tel: +86 29 87092102. Fax: +86 29 87092164. e-mail: hjh19732008@yahoo.com.cn or liqingwangysu@yahoo.com.cn
[2]College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, P.R. China.

the proliferation SSCs derived from DBA/2 mice, but not for other mouse strains (Kanatsu-Shinohara *et al.*, 2003). Although the growth requirements of mouse SSCs still remain unclear, several laboratories have attempted to culture SSCs for long or short time periods *in vitro* (Jeong *et al.*, 2003; Kanatsu-Shinohara *et al.*, 2003; Nagano *et al.*, 2003).

Germ cells and somatic cells, such as Sertoli cells and Leydig cells, in the testis can support spermatogenesis via interactions between these cells and actions of hormones secreted by the pituitary gland and the testis (Jegou, 1993; Parvinen *et al.*, 1986; Skinner, 1991). It has been claimed that some growth factors have important functions in mammalian testis. Insulin-like growth factors (IGFs) can stimulate mitotic DNA synthesis (Soder *et al.*, 1992) and the stem-cell factor (SCF)/c-kit system is essential for survival (Packer *et al.*, 1995; Hakovirta *et al.*, 1999), proliferation and for differentiation of germ cells (Yoshinaga *et al.*, 1991; Manova *et al.*, 1993; Vincent *et al.*, 1998; Sette *et al.*, 2000). However, little information is known about the effects of testicular interstitial fluid on mouse SSCs proliferation *in vitro*. In this study, an original, undefined and complex culture system was designed to culture mouse SSCs in order to obtain more information about the use of testicular interstitial fluid as a supplement for proliferation of mouse SSCs *in vitro*.

Materials and methods

All chemical agents used in the study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental animals

Eighty Institute for Cancer Research (ICR) male mice (6-day-old) were obtained from the Experimental Animal Center of Medical College of Xi'an Jiaotong University, China for use in the study.

Preparation of testicular interstitial fluid

Forty testes were excised from mice and cut into small fragments. Subsequently, the testicular interstitial fluid was extracted by tissue homogenization.

Preparation of culture medium

Components of M1 medium were Dulbecco's modified Eagle's medium (DMEM), 4 mol/l L-glutamine, 1% non-essential amino acids, 4×10^{-2} mg/ml gentamicin and 10% fetal bovine serum (FBS). M2 medium was composed of DMEM, 4 mol/l L-glutamine, 1% non-essential amino acids, 4×10^{-2} mg/ml gentamicin, 10% FBS and testicular interstitial fluid. The final concentrations of testicular interstitial fluid in M2 medium were 0, 5, 10, 20, 30 or 40% (v/v), respectively.

Isolation and purification of mouse SSCs

Two-step enzymatic digestion was used to prepare single cell suspensions from testis tissues (Herrid *et al.*, 2007). The dispersed cells were filtrated with a four-hundred-eye mesh and rinsed twice with DMEM, then the pellet was suspended in M1 medium. The cells were cultured by a two-step differential plating process to enrich for SSCs. Briefly, 10^6 cells/ml were cultured in 60-mm dishes for 12 h at 37°C in an atmosphere of 5% CO_2 in air, then the non-adherent cells were transferred to a new plate and cultured for 4 h under the same conditions. Suspended cells were collected and divided into six aliquots. The first aliquot was cultured *in vitro* as a control; the other five aliquots were cultured in parallel in 48-well plates at 5×10^4 cells per well in M2 medium. The cells were sub-cultured every day by replenishing half the medium. The cell suspension was placed into cell culture flasks and maintained at 37°C in a humidified 5% CO_2 and 95% air atmosphere. As a result of the differential plating, a final proportion of about 80–85% SSCs was gained by eliminating adherent Sertoli cells every 6–8 h. The medium was changed every 2–3 days.

Alkaline phosphatase (AKP) assay

Mouse SSCs were fixed in 4% paraformaldehyde and then stained with NBT/BCIP AKP substrate. The staining reaction was stopped after 20–40 min incubation in light by rinsing the cells with phosphate-buffered saline (PBS). Stained cells were observed and photographed under an inverted phase contrast microscope (Nikon Imaging Sales Co Ltd., Tokyo, Japan).

RT-PCR

Total cellular RNA was extracted from mouse SSCs and Sertoli cells using the RNA Simple Total RNA Kit (TianGen Co. Ltd, China). Reverse-transcription was performed using the SuperScript™ First-Strand Synthesis System and cDNAs were used to analyze the expression of SSC-specific genes by PCR. β-*Actin* was used as the housekeeping gene. The primer sequences and GenBank accession numbers are as follows: *Ngn3* (GenBank No. NM_009719.6) Forward Primer: 5′-TTGGCACTCAGCAAACAGC-3′, Reverse Primer: 5′-TCCCTTTCCACTAGCACCC-3′, 467 bp; *Oct4* (GenBank No. NM_013633.2) Forward Primer: 5′-CCCCAATGCCGTGAAGTT-3′, Reverse Primer: 5′-GAAAGGTGTCCCTGTAGCC-3′, 556 bp; β-*actin*

https://doi.org/10.1017/S0967199413000142 Published online by Cambridge University Press

(GenBank No. NM_007393.3) Forward Primer: 5′-GCCTTCCTTCTTGGGTAT-3′, Reverse Primer: 5′-CCTTCACCGTTCCAGTTT-3′, 549 bp; *TH2B* (GenBank No. NM_175663.1) Forward Primer: 5′-CGGTAAAGGGTGCTACTAT-3′, Reverse Primer: 5′-CACTTGTTTCAGCACCTTA-3′, 137 bp; *Integrin alpha 6* (GenBank No. NM_008397.3) Forward Primer: 5′-ATGATGAAAGTCTCGTGC-3′, Reverse Primer: 5′-CATAGCCAAACGAGGAAG-3′, 222 bp; *Integrin beta 1* (GenBank No. NM_010578.2) Forward Primer: 5′-TTGATGAATGAAATGAGGAG-3′, Reverse Primer: 5′-TCCAGATATGCGTTGCTG-3′, 225 bp; and *Sycp3* (GenBank No. NM_011517.2) Forward Primer: 5′-TCAGAGCCAGAGAATGAAAG-3′, Reverse Primer: 5′-CTGCTGAGTTTCCATCATAAC-3′, 163 bp. The reaction products were separated and visualized by 1.0% agarose gel electrophoresis.

Indirect immunofluorescence cell analysis

The mouse SSCs obtained were evaluated by staining with CD9, which is a marker for undifferentiated type A spermatogonia (Abu Elhija *et al.*, 2012). Briefly, cells were fixed in 4% paraformaldehyde for 15 min and then were rinsed three times in PBS plus 0.1% Tween-20. Subsequently, the cells were re-suspended in PBS plus bovine serum albumin (BSA) for 1 h at 37°C. The cells were then incubated with anti-CD9 antibody (final concentration 1:1000) for 12 h at 37°C. After three washes in PBS, cells were incubated with the Cy3 AffiniPure Goat Anti-Rabbit IgG (H+L) (final concentration 1:1000) for 1 h at 37°C. Finally, the solution was incubated with Hoechst stain (1:800) for 8 min and then washed three times with PBS. Cells were re-suspended in 0.5 ml PBS–BSA. Fluorescence was monitored using the 8- and 644-nm double band-pass filter and fluorescence microscopy (Nikon Imaging Sales Co Ltd., Tokyo, Japan).

Proliferation and diameter of SSCs colony assay

The mouse SSCs obtained were treated with 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reagent for 4 h at 37°C to measure cell proliferation and placed in a shaker for 20 min. For quantitation, dimethyl sulfoxide (DMSO) was added to dissolve water-insoluble blue-violet MTT formazan formed in the cells. The diameters of cell colonies were measured using the microscope software.

Statistical analysis

All statistical analyses were performed using Statistical Product and Service Solutions software (SPSS 11.5 for Windows; SPSS, Chicago, IL, USA). For culture assays of the mouse SSCs with the testicular interstitial fluid *in vitro*, each data point was represented by the average of three separate experiments and presented as mean ± standard deviation (SD). Statistical significance among the proliferation rate and diameter of SSCs colony were determined using Duncan's multiple range test and by analysis of variance (ANOVA). A P-value <0.05 indicated statistical significance.

Results

Characterization of SSCs during short-term culture

Isolated cells were enriched by the anchorage velocity-dependent separation method. After 6–8 days of culture, cell colonies formed in the dishes (Fig. 1). SSC cells appeared round, and began to divide and proliferate at 2 days and cell colonies emerged after culture for 6 days (Fig. 1).

Alkaline phosphatase (AKP) assay

The control group in the assay was represented by purified Sertoli cells. Stem cells stained bluish violet by AKP staining. In two images (Fig. 2*A*, *B*), SSCs and Sertoli cells were clear differentiated; SSCs were round and stained bluish violet, while Sertoli cells had an irregular form and stained minimally. The AKP assay and cell colony imaging (Fig. 1) demonstrated that the round cells could be considered to be SSC colonies.

Characteristic genes expression

Some genes characteristic of germ cells were selected to identify SSCs and haploid male germ cells. The *Ngn3* gene is typical of SSCs (Yoshida *et al.*, 2004) and *Oct4* expression marks the pluripotency and self-renewal of stem cells (Loh *et al.*, 2006). The β-*actin* gene was used as the housekeeping gene for SSCs and Sertoli cells (Thellin *et al.*, 1999). *Integrin alpha 6* and *Integrin beta 1* gene products are expressed as surface markers on SSCs (Shinohara *et al.*, 1999). Expression of *Sycp3* and *TH2B* indicates the presence of haploid male germ cells (Lim *et al.*, 2010). The mouse SSCs obtained expressed *OCT4*, *Integrin alpha 6*, *Integrin beta 1*, β-*actin*, *Ngn 3* (Fig. 3). Sertoli cells only expressed β-*actin*, data not shown.

Indirect immunofluorescence cell analysis

It is usual to consider that As (a single) and Apr (a paired) spermatogonia have stem-cell properties. As no specific markers have been reported, CD9 was selected as the surface maker for SSCs. The results of indirect immunofluorescence analysis of Sertoli cells and SSCs are shown in Fig. 4. It was clear that the SSCs colonies expressed CD9 on their cell surfaces after culture for 3–5 days (Fig. 4). Although Sertoli

https://doi.org/10.1017/S0967199413000142 Published online by Cambridge University Press

Table 1 The effects of testes liquid on mouse spermatogonial stem cells (SSCs) proliferation *in vitro*

Testicular interstitial fluid	Proliferation rate of SSCs (%)	Diameter of SSCs colonies (μm)
Control groups	10.15 ± 0.58^{a}	109 ± 15.6^{a}
5% testicular interstitial fluid	26.65 ± 1.62^{b}	167 ± 26.8^{b}
10% testicular interstitial fluid	42.83 ± 0.23^{c}	178 ± 35.9^{b}
20% testicular interstitial fluid	67.74 ± 2.31^{d}	209 ± 30.5^{c}
30% testicular interstitial fluid	72.53 ± 0.68^{e}	249 ± 29.7^{d}
40% testicular interstitial fluid	43.67 ± 1.59^{f}	181 ± 45.8^{b}

Note: Values in the same row with different letters *a–f* indicate significant difference ($P < 0.05$).

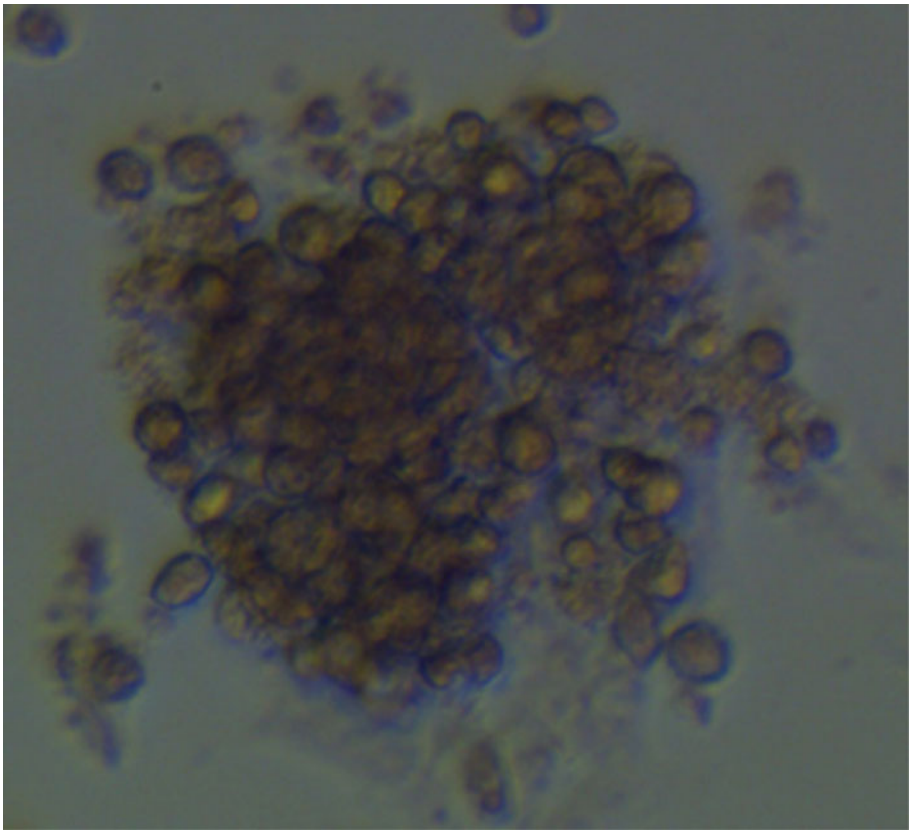

Figure 1 Cell colonies (×250 magnification).

cells also expressed CD9, staining showed a small spherical nucleus with a thick rim of cytoplasm (Fig. 5). In contrast, SSCs had a large spherical nucleus with a thin rim of cytoplasm (Fig. 4*A*, *C*. Therefore we demonstrated here again that the cell colonies were SSCs.

Effects of testicular interstitial fluid on mouse SSCs proliferation *in vitro*

After overnight culture, most SSCs were scattered singly and some had begun to divide. It is clearly shown in Table 1 that the optimal proliferation rate and the diameter of the SSC clusters were 72.53% and 249 μm, respectively, in the 30% testicular interstitial fluid treated group after 8 days, values that were significantly higher ($P < 0.05$) than other groups. This finding demonstrated that medium that contained 30% testicular interstitial fluid could significantly promote mouse SSC proliferation *in vitro*.

Discussion

Testicular interstitial fluid is a good resource for SSCs culture *in vitro* because it is rich in growth factors and able to support a specific niche for SSCs (Yoshinaga *et al.*, 1991; Soder *et al.*, 1992; Vincent

https://doi.org/10.1017/S0967199413000142 Published online by Cambridge University Press

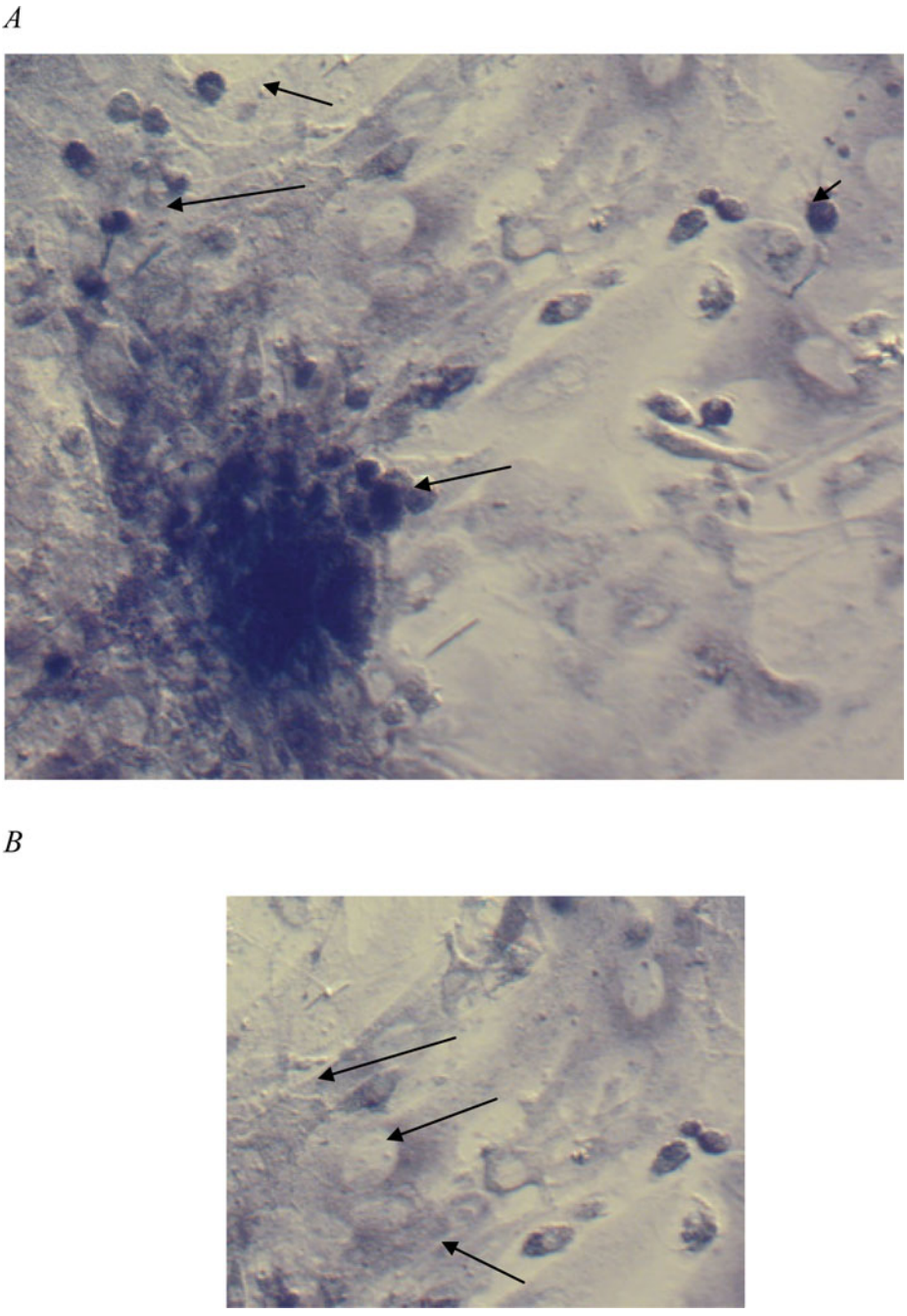

Figure 2 (*A*) Cells with alkaline phosphatase staining (×200 magnification). Arrows indicate that the cells stained bluish violet were considered to be spermatogonial stem cells (SSCs). (*B*) Cells with alkaline phosphatase staining (×200). The arrows indicate the Sertoli cells. The SSCs stained bluish violet (and were round), but the Sertoli cells were minimally stained (and were irregular in shape).

et al., 1998; Hakovirta *et al.*, 1999; Sette *et al.*, 2000). In particular, it is assumed that the early differentiating spermatogonia and SSCs emerge at the time when germ cells locate in the lumen of immature seminiferous tubules in the neonatal testis (de Rooij, 2006). Hence, it is possible to efficiently recover SSCs from testis by a differential plating method. The optimal proliferation rate and diameter of the SSCs colony was 72.53% and 249 μm, respectively, when the culture medium was supplemented to 30% with the testicular interstitial fluid.

AKP assay, RT-PCR and indirect immunofluorescence cell analysis were performed to verify the mouse SSCs in the study. AKP is a pluripotency cell marker (Neri *et al.*, 2007; Park *et al.*, 2008). The AKP-positive cells have the properties of stem cells and are stained

https://doi.org/10.1017/S0967199413000142 Published online by Cambridge University Press

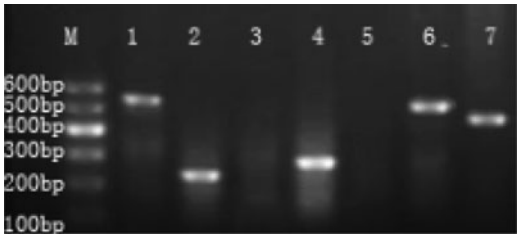

Figure 3 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis. The characteristic genes of spermatogonial stem cells (SSCs). The M lane indicates the DNA marker; lane 1 indicates *Oct4*; lane 2 is *Integrin alpha 6*; lanes 3, 4, 5, 6 and 7 indicate the expression of *TH2B*, *Integrin beta 1*, *Scyp3*, β-*actin* and *Ngn 3*, respectively.

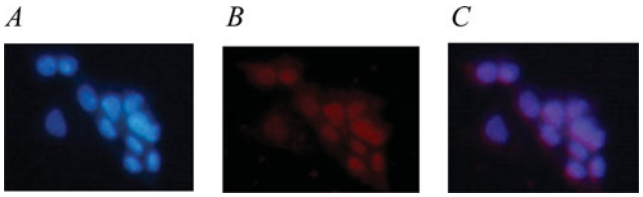

Figure 4 Spermatogonial stem cells (SSCs) were identified by CD9. (*A*) SSCs were stained with Hoechst (×200). (*B*) SSCs were defined by CD9 indirect immunofluorescence staining (×200 magnification). (*C*) SSCs were defined by CD9 and by Hoechst stain combined with indirect immunofluorescence staining (×200), (*C*) the merging of images (*A*) and (*B*).

bluish violet. Our results were similar to those in previous studies (Guan *et al.*, 2006; Goel *et al.*, 2007). *Oct4*, *Integrin alpha 6*, *Integrin beta 1*, *and Ngn3* genes were selected to identify the SSCs and the Sertoli cells (Shinohara *et al.*, 1999). In male mice, the *Oct-4* gene is expressed before spermatogenesis and is confined to type A spermatogonia (Feng *et al.*, 2002). The *Ngn3* gene is predominantly expressed in the As, Apr and Aal stages of c-Kit-negative spermatogonia in adults and the c-Kit-negative fraction of the pre-pubertal prespermatogonia (Yoshida *et al.*, 2004). *Integrin alpha 6* and *Integrin beta 1* are considered to be surface markers for SSCs (Shinohara *et al.*, 1999). Thus, the expression of the *Ngn3*, *Oct4*, *Integrin alpha 6* and *Integrin beta 1* genes can be considered as a marker of SSCs and Sertoli cells. In this study, the mouse SSCs obtained expressed *OCT4*, *Integrin alpha 6*, *Integrin beta 1*, β-*actin* and *Ngn 3* (Fig. 3). The expression of *Sycp3* and *TH2B* is considered the specific marker of haploid male germ cells (Lim *et al.*, 2010; Li *et al.*, 2011), thus the expression of the *TH2B* and *Scyp3* genes can be considered to indicate differentiated SSCs and haploid male germ cells. In Fig. 3, we can see that the SSCs did not express the *TH2B* and *Scyp3*, which indicated that the SSCs had not started to differentiate. As no specific markers were reported, CD9 was selected as the surface maker of SSCs. It was clear that the cell colonies expressed CD9 on the cell surface after culture for 3–5 days (Fig. 4). Although the Sertoli cells were also expressed CD9, they showed a small spherical nucleus with a thick rim of cytoplasm (Fig. 5). In contrast, mouse SSCs had a large spherical nucleus with a thin rim of cytoplasm (Fig. 4*A*, *C*), whose morphology was the same as the mouse SSCs. Therefore, it was obvious that the cell colonies were indeed undifferentiated mouse SSCs.

The specific biochemical and cytoarchitectural microenvironment in the adluminal compartment where germ cells proliferate and differentiate are constituted the region for tissue remodeling and the production of Sertoli cell factors during testis development (Griswold, 1995). Until a niche is available, only one in two daughter cells can remain and the other cell will be committed to differentiate (Schofield, 1978). The culture system for SSCs consisted of serum medium and mitosis inactivated Sertoli cell feeders; purified mouse SSCs were seeded on the feeders at densities of $6–10 \times 10^4$ cells/ml in this study. It was demonstrated that proliferation of the mouse

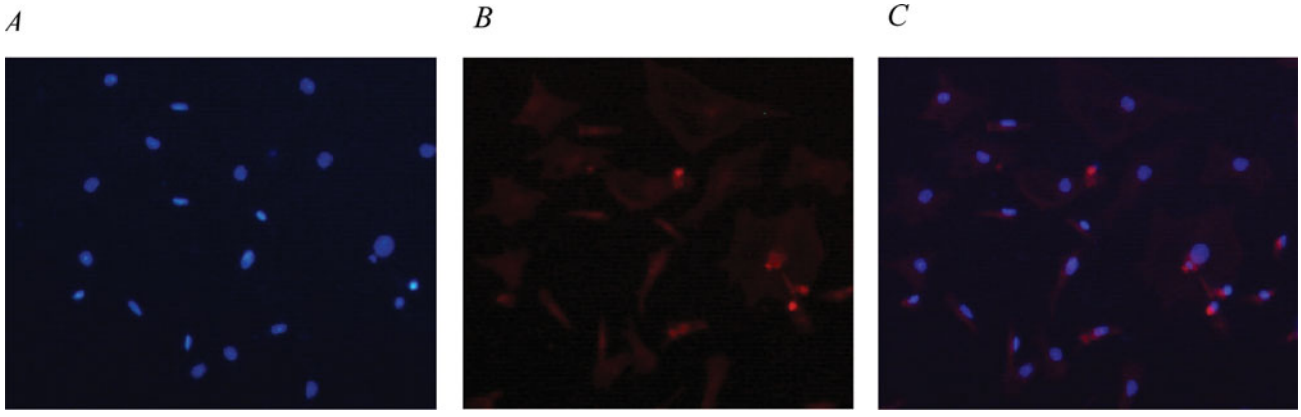

Figure 5 Sertoli cells were identified by CD9. (*A*) Sertoli cells were stained with Hoechst (×400). (*B*) Sertoli cells were detected by CD9 by indirect immunofluorescence staining (×400). (C) Sertoli cells were detected by CD9 by Hoechst combined with indirect immunofluorescence staining (×400), (*C*) is the merging of images (*A*) and (*B*). The control group was Sertoli cells.

https://doi.org/10.1017/S0967199413000142 Published online by Cambridge University Press

SSCs could be largely promoted by supplementing the M2 medium with 30% testicular interstitial fluid. The optimal diameter of the mouse SSCs colony was 249 μm at 8 days. Some factors have an important role in germ cell proliferation in the testis. Neuregulin 1 (NRG1) belonging to the epidermal growth factor (EGF) family can induce growth and differentiation of some types of cells during cultivation (Falls, 2003); SCF is produced by the Sertoli cells (Rossi *et al.*, 1993) and can promote germ cell proliferation and survival (Tajima *et al.*, 1991); interleukin (IL)-α has been considered to stimulate the proliferation of germ cells, Sertoli cells and peritubular cells (Pollanen *et al.*, 1989; Parvinen *et al.*, 1991; Svechnikov *et al.*, 2001; Petersen *et al.*, 2002); testosterone is necessary for the completion of spermatogenesis and maintenance of male reproductive function. Testis is rich in growth factors such as acidic fibroblast growth factor (aFGF), nerve growth factor (NGF), seminiferous growth factor (SGF), IGF-1, and transforming growth factors (TGF) alpha and beta (Braunhut *et al.*, 1990; Skinner, 1991). According to our results, it is hypothesized that culture medium supplemented with 30% testicular interstitial fluid contains the appropriate concentration of hormones and growth factors to promote mouse SSC growth. In the testes of 6-old-day mice, this niche is available for SSCs maintainance rather than differentiation. These original, undefined and complex testicular interstitial fluid conditions make it possible to culture SSCs *in vitro*, but more details are needed to expound this result.

The successful culture of SSCs *in vitro* is beneficial to SSC research. The present study indicated that culture medium containing 30% of the testicular interstitial fluid could significantly enhance mouse SSCs proliferation *in vitro*. The use of this culture system is valuable for subsequent experiments, such as in pluripotency of SSCs, production of genetically modified animals, gene therapy, and so on. However, more research is need to evaluate and understand the precise physiological role of testicular interstitial fluid in cultured SSCs *in vitro*.

Acknowledgements

This research was supported by the Youth Extra Fund of Northwest A&F University (Z111020905), the Special Research Subsidy Project of Northwest A&F University (07ZR002) and the Basic Scientific Research Expense of Sci-Tech Innovation Major Project of Northwest A&F University (QN2011061).

References

Abu Elhija, M., Lunenfeld, E., Schlatt, S. & Huleihel, M. (2012). Differentiation of murine male germ cells to spermatozoa in a soft agar culture system. *Asian J. Androl.* **14**, 285–93.

Araki, Y., Sato, T., Katagiri, K., Kubota, Y., Araki, Y. & Ogawa, T. (2010). Proliferation of mouse spermatogonial stem cells in microdrop culture. *Biol. Reprod.* **83**, 951–7.

Braunhut, S.J., Rufo, G.A., Ernisee, B.J., Zheng, W.X. & Bellve, A.R. (1990). The seminiferous growth factor induces proliferation of TM4 cells in serum-free medium. *Biol. Reprod.* **42**, 639–48.

Brinster, R.L. & Nagano, M. (1998). Spermatogonial stem cell transplantation, cryopreservation and culture. *Semin. Cell Dev. Biol.* **9**, 401–9.

Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol. Rev.* **52**, 198–236.

de Rooij, D.G. (2006). Rapid expansion of the spermatogonial stem cell tool box. *Proc. Natl. Acad. Sci. USA* **103**, 7939–40.

Falls, D.L. (2003). Neuregulins: functions, forms, and signaling strategies. *Exp. Cell Res.* **284**, 14–30.

Feng, L.X., Chen, Y.L., Dettin, L., Pera, R.A.R., Herr, J.C., Goldberg, E. & Dym, M. (2002). Generation and *in vitro* differentiation of a spermatogonial cell line. *Science* **297**, 392–5.

Goel, S., Sugimoto, M., Minami, N., Yamada, M., Kume, S. & Imai, H. (2007). Identification, isolation, and *in vitro* culture of porcine gonocytes. *Biol. Reprod.* **77**, 127–37.

Griswold, M.D. (1995). Interactions between germ-cells and Sertoli cells in the testis. *Biol. Reprod.* **52**, 211–6.

Guan, K., Nayernia, K., Maier, L.S., Wagner, S., Dressel, R., Lee, J.H., Nolte, J., Wolf, F., Li, M. & Engel, W. (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* **440**, 1199–203.

Hakovirta, H., Yan, W., Kaleva, M., Zhang, F.P., Vanttinen, K., Morris, P.L., Soder, M., Parvinen, M. & Toppari, J. (1999). Function of stem cell factor as a survival factor of spermatogonia and localization of messenger ribonucleic acid in the rat seminiferous epithelium. *Endocrinology* **140**, 1492–8.

Herrid, M., Davey, R.J. & Hill, J.R. (2007). Characterization of germ cells from pre-pubertal bull calves in preparation for germ cell transplantation. *Cell Tissue Res.* **330**, 321–9.

Huckins, C. (1971). The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *Anat. Rec.* **169**, 533–57.

Jegou, B. (1993). The Sertoli germ-cell communication-network in mammals. *Int Rev. Cytol* **147**, 25–96.

Jeong, D.K., McLean, D.J. & Griswold, M.D. (2003). Long-term culture and transplantation of murine testicular germ cells. *J. Androl.* **24**, 661–9.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S. & Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol. Reprod.* **69**, 612–6.

Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., Yoshida, S., Toyokuni, S., Lee, J.Y., Ogura, A. & Shinohara, T. (2007). Leukemia inhibitory factor enhances formation of germ cell colonies in neonatal mouse testis culture. *Biol. Reprod.* **76**, 55–62.

Kubota, H., Avarbock, M.R. & Brinster, R.L. (2004). Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. *Biol. Reprod.* **71**, 722–31.

Lee, D.R., Parks, J.E., Lim, J.J., Yoon, H., Ko, J.J. & Kim, K.S. (2003). Regulation of the proliferation and differentiation of mouse spermatogonial stem cells by LIF/bFGF and FSH during *in vitro* culture. *Fertil. Steril.* **80**, S279.

Li, X.C., Bolcun-Filas, E. & Schimenti, J.C. (2011). Genetic evidence that synaptonemal complex axial elements govern recombination pathway choice in mice. *Genetics* **189**, 71–U668.

Lim, J.J., Sung, S.Y., Kim, H.J., Song, S.H., Hong, J.Y., Yoon, T.K., Kim, J.K., Kim, K.S. & Lee, D.R. (2010). Long-term proliferation and characterization of human spermatogonial stem cells obtained from obstructive and non-obstructive azoospermia under exogenous feeder-free culture conditions. *Cell Prolif.* **43**, 405–17.

Loh, Y.H., Wu, Q., Chew, J.L., Vega, V.B., Zhang, W.W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., Wong, K. Y., Sung, K. W., Lee, C. W. H., Zhao, X. D., Chiu, K. P., Lipovich, L., Kuznetsov, V. A., Robson, P., Stanton, L. W., Wei, C. L., Ruan, Y. J., Lim, B. & Ng, H. H. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.* **38**, 431–40.

Manova, K., Huang, E.J., Angeles, M., De Leon, V., Sanchez, S., Pronovost, S.M., Besmer, P. & Bachvarova, R.F. (1993). The expression pattern of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in oocyte growth and in proliferation of spermatogonia. *Dev. Biol.* **157**, 85–99.

Morrison, S.J., Uchida, N. & Weissman, I.L. (1995). The biology of hematopoietic stem cells. *Annu Rev. Cell Dev. Biol.* **11**, 35–71.

Nagano, M., Avarbock, M.R., Leonida, E.B., Brinster, C.J. & Brinster, R.L. (1998). Culture of mouse spermatogonial stem cells. *Tissue Cell* **30**, 389–97.

Nagano, M., Ryu, B.Y., Brinster, C.J., Avarbock, M.R. & Brinster, R.L. (2003). Maintenance of mouse male germ line stem cells *in vitro*. *Biol. Reprod.* **68**, 2207–14.

Neri, T., Monti, M., Rebuzzini, P., Merico, V., Garagna, S., Redi, C.A. & Zuccotti, M. (2007). Mouse fibroblasts are reprogrammed to Oct-4 and Rex-1 gene expression and alkaline phosphatase activity by embryonic stem cell extracts. *Cloning Stem Cells* **9**, 394–406.

Nolte, J., Michelmann, H.W., Wolf, M., Wulf, G., Nayernia, K., Meinhardt, A., Zechner, U. & Engel, W. (2010). PSCDGs of mouse multipotent adult germline stem cells can enter and progress through meiosis to form haploid male germ cells *in vitro*. *Differentiation* **80**, 184–94.

Oatley, J.M., Avarbock, M.R. & Brinster, R.L. (2007). Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on src family kinase signaling. *J. Biol. Chem.* **282**, 25842–51.

Packer, A.I., Besmer, P. & Bachvarova, R.F. (1995). Kit-ligand mediates survival of type-a spermatogonia and dividing spermatocytes in postnatal mouse testes. *Mol. Reprod. Dev.* **42**, 303–10.

Park, I.H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H.G., Ince, T.A., Lerou, P.H., Lensch, M.W. & Daley, G.Q. (2008). Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451**, 141–6.

Parvinen, M., Vihko, K.K. & Toppari, J. (1986). Cell interactions during the seminiferous epithelial cycle. *Int. Rev. Cytol.* **104**, 115–51.

Parvinen, M., Soder, O., Mali, P., Froysa, B. & Ritzen, E.M. (1991). *In vitro* stimulation of stage-specific deoxyribonucleic acid synthesis in rat seminiferous tubule segments by interleukin-1 alpha. *Endocrinology* **129**, 1614–20.

Petersen, C., Boitani, C., Froysa, B. & Soder, O. (2002). Interleukin-1 is a potent growth factor for immature rat Sertoli cells. *Mol. Cell Endocrinol.* **186**, 37–47.

Pollanen, P., Soder, O. & Parvinen, M. (1989). Interleukin-1 alpha stimulation of spermatogonial proliferation *in vivo*.*Reprod. Fertil. Dev.* **1**, 85–7.

Rossi, P., Dolci, S., Albanesi, C., Grimaldi, P., Ricca, R. & Geremia, R. (1993). Follicle-stimulating hormone induction of steel factor (SLF) mRNA in mouse Sertoli cells and stimulation of DNA synthesis in spermatogonia by soluble SLF. *Dev. Biol.* **155**, 68–74.

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* **4**, 7–25.

Sette, C., Dolci, S., Geremia, R. & Rossi, P. (2000). The role of stem cell factor and of alternative c-kit gene products in the establishment, maintenance and function of germ cells. *Int. J. Dev. Biol.* **44**, 599–608.

Shinohara, T., Avarbock, M.R. & Brinster, R.L. (1999). Beta(1)- and alpha(6)-integrin are surface markers on mouse spermatogonial stem cells. *Proc Natl Acad Sci USA* **96**, 5504–5509.

Skinner, M.K. (1991). Cell-cell interactions in the testis. *Endocr. Rev.* **12**, 45–77.

Soder, O., Bang, P., Wahab, A. & Parvinen, M. (1992). Insulin-like growth factors selectively stimulate spermatogonial, but not meiotic, deoxyribonucleic acid synthesis during rat spermatogenesis. *Endocrinology* **131**, 2344–50.

Spradling, A., Drummond-Barbosa, D. & Kai, T. (2001). Stem cells find their niche. *Nature* **414**, 98–104.

Svechnikov, K.V., Sultana, T. & Soder, O. (2001). Age-dependent stimulation of Leydig cell steroidogenesis by interleukin-1 isoforms. *Mol. Cell Endocrinol.* **182**, 193–201.

Tajima, Y., Onoue, H., Kitamura, Y. & Nishimune, Y. (1991). Biologically active kit ligand growth factor is produced by mouse Sertoli cells and is defective in Sld mutant mice. *Development* **113**, 1031–1035.

Tegelenbosch, R.A.J. & Derooij, D.G. (1993). A quantitative study of spermatogonial multiplication and stem-cell renewal in the C3H/101 F1-hybrid mouse. *Mutat. Res.* **290**, 193–200.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A. & Heinen, E. (1999). Housekeeping genes as internal standards: use and limits. *J. Biotechnol.* **75**, 291–5.

Uchida, N., Fleming, W.H., Alpern, E.J. & Weissman, I.L. (1993). Heterogeneity of hematopoietic stem-cells. *Curr. Opin. Immunol.* **5**, 177–84.

Vincent, S., Segretain, D., Nishikawa, S., Nishikawa, S.I., Sage, J., Cuzin, F. & Rassoulzadegan, M. (1998). Stage-specific expression of the Kit receptor and its ligand (KL) during male gametogenesis in the mouse: a Kit-KL interaction critical for meiosis. *Development* **125**, 4585–93.

Yoshida, S., Takakura, A., Ohbo, K., Abe, K., Wakabayashi, J., Yamamoto, M., Suda, T. & Nabeshima, Y. (2004). Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. *Dev. Biol.* **269**, 447–58.

Yoshinaga, K., Nishikawa, S., Ogawa, M., Hayashi, S., Kunisada, T. & Fujimoto, T. (1991). Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. *Development* **113**, 689–99.