TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2014) 38: 693-698
© TÜBİTAK
doi:10.3906/vet-1402-70

Study of insulin-like growth factor 1 effects on bovine type A spermatogonia proliferation and viability

Babak QASEMI PANAHI[1,*], Parviz TAJIK[2], Mansoureh MOVAHEDIN[3], Gholamali MOGHADDAM[1], Mohammad Hossein GERANMAYEH[4]

[1]Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
[2]Department of Clinical Science, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[3]Department of Anatomy, Faculty of Medical Science, University of Tarbiat Modares, Tehran, Iran
[4]Department of Physiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Received: 18.02.2014 • **Accepted:** 28.06.2014 • **Published Online:** 24.10.2014 • **Printed:** 21.11.2014

Abstract: Spermatogonial stem cells (SSCs) are the best candidates for exploitation in genetic improvement of cattle herds. In fact, SSCs transmit genetic information to future generations. In vitro improvement in proliferation and frozen-thawed SSCs numbers is therefore needed. In the present study, bovine SSCs were cocultured with Sertoli cells, with or without insulin-like growth factor 1 (IGF-1). During 16 days of incubation, 100 ng/mL IGF-1 showed higher efficacy in increasing the numbers and diameters of SSCs colonies than 40 and 150 ng/mL concentrations. Furthermore, 100 ng/mL IGF-1 increased the frozen/thawed cells' viability rate. Overall, these data demonstrate that usage of 100 ng/mL IGF-1 in vitro improves proliferation and postfreezing viability rate of SSCs.

Key words: Spermatogonial stem cells, proliferation, IGF-1, bovine

1. Introduction

Spermatogonial stem cells (SSCs) are considered an undifferentiated spermatogonial subpopulation that has self-renewal and differentiation potential. SSCs are able to continue spermatogenesis after transplantation into infertile testes (1). Spermatogenesis in mammals is a procedure for transmitting genetic information to future generations, which, after adulthood, continues throughout the male life. Spermatozoa are the final product of this process (2). Cell culture techniques' development has made an opportunity for germ-line modification and improvement in a broad range of species (3). Transplantation of transfected SSCs into seminiferous tubules of bull calves testes can be used for production of transgenic animals. After transplantation they migrate to the basal layer of tubules and initiate proliferation and spermatogenesis (4). Thus, increasing proliferation and viability rate of SSCs in vitro facilitates great successes in the field of genetic improvement of cattle herds. Type A spermatogonia can be obtained in large numbers in prepubertal bulls (4,5). Isolation of type A spermatogonia is performable by 2-enzyme digestion of calf testes, which gives 65%-87% pure cell populations (6). In the past, researchers used different culture conditions and cryopreservation methods for improvement of proliferation and survival of spermatogonia (6–8).

* Correspondence: babakpanahi98@yahoo.com

Insulin-like growth factor 1 (IGF-1) consists of a peptide chain of 70 amino acid residues with 3 disulfide bridges that is homologous to proinsulin (9,10). Leydig cells in normal testes secrete IGF-1, which regulates self-renewal and pluripotency of SSCs via IGF-1 R-mediated PI3K/Akt signaling (11). Synthetic IGF-1 inhibits apoptosis (12,13) and it has mitogenic effects on cells (14,15).

In this context we examined the effects of IGF-1 on proliferation and viability of bovine type A spermatogonia. Appropriate doses of IGF-1 in SSC cultures result in optimal size and numbers of colonies for various experiments in this field.

2. Materials and methods

2.1. Experimental animals

In this survey, 5 male Holstein Friesian calves of 3 months old were used. The research was conducted in accordance with National Research Council guidelines. Calves were sedated with xylazine (0.1 mg/kg of body weight, i.m.) and received a 3-mL injection of 2% lidocaine 20 min before surgery. Respiration rate, heart rate, and rectal temperature were monitored before and after recovery from analgesia. Calves were given flunixin meglumine (2.2 mg/kg of body weight, i.m.) for 3 days after surgery.

2.2. Germ cell and Sertoli cell collection

Testicular biopsy samples were placed on ice and transferred to the laboratory within 2 h. The testes samples were minced into small pieces and suspended in Dulbecco's modified Eagle's Medium (DMEM; Sigma, St Louis, MO, USA), supplemented with 160.7 mM $NaHCO_3$ (Sigma), single-strength nonessential amino acids, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 40 µg mL^{-1} gentamicin (all from GIBCO, Carlsbad, CA, USA). SSC isolation was prepared by the method previously described by Van Pelt et al. (16). In brief, testes fragments were rinsed in DMEM containing 1 mg mL^{-1} collagenase, 1 mg mL^{-1} trypsin, 1 mg mL^{-1} hyaluronidase type II, and 5 µg mL^{-1} DNAse I (all from Sigma) and were incubated at 37 °C for 60 min. Mechanical dissociation was also done by a little pipetting for better testicular tissue digestion. After removal of interstitial cells by 3 washes with DMEM, seminiferous fragments were incubated in DMEM containing collagenase, hyaluronidase, and DNAse for 45 min as described above. By centrifugation at 30 × *g* for 2 min, the cells were separated from the remaining tubule fragments. Nylon filters of 77 and 55 mm were used, and then isolated cells were cultured.

2.3. Sertoli cell isolation

Following the second enzymatic digestion, *Datura stramonium* agglutinin (DSA; Sigma) was used for isolation of Sertoli cells. Briefly, 25-cm^2 flasks were coated with 5 µg mL^{-1} DSA in phosphate-buffered saline (PBS) at 37 °C for 1 h. After 3 washes with DMEM containing 0.5% bovine serum albumin (BSA; Sigma), the suspensions of cells obtained by enzymatic digestion were placed in coated flasks and incubated for 1 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After the incubation period, nonadherent cells were collected. After 3 days the Sertoli cells formed a confluent layer.

2.4. Spermatogonial cells' coculture with Sertoli cells

After achieving appropriate confluency of Sertoli cells, SSCs were cocultured for 2 weeks. DMEM supplemented with 160.7 mM NaHCO3, 10% FBS, 100 ng mL^{-1} glial cell line-derived neurotrophic factor (GDNF), single-strength nonessential amino acids, 100 IU mL^{-1} penicillin, 100 µg mL^{-1}, streptomycin and 40 µg mL^{-1} gentamicin was used.

After 1 week of coculturing, colonies of SSCs were seen. The cells were then passaged and cultured in 24-well plates in 4 groups including: the control, group 1 (40 ng/mL IGF-1), group 2 (100 ng/mL IGF-1), and group 3 (150 ng/mL IGF-1). Culture medium together with the mentioned doses of IGF-1 were refreshed every 3 days.

2.5. Colony assay

Determination of numbers and diameter of SSC-derived colonies was carried out on day 7 and the following 10th, 13th, and 16th days (8). This assessment was prepared with an inverted microscope (Olympus, Tokyo, Japan) equipped with an ocular grid.

2.6. Identification of spermatogonial and Sertoli cells

For immunocytochemical analysis, 1–2 × 10^4 cells were expanded in each well of 8-well glass slides (Marienfeld, Lauda-Königshofen, Germany) overnight in a humidified incubator followed by washing with prewarmed medium. After drying for 15 min, cells were fixed and permeabilized in acetone for 2 min at –20 °C and kept at 4 °C for 30 min until slides were completely dried. Slides were then washed 3 times (3 × 3 min) with Tris-buffered saline, pH 7.4, containing 5% BSA (TBS/BSA). Blocking was performed with 5% horse serum in TBS for 10 min and goat anti-Oct-4 polyclonal antibody (Abcam, Cambridge, UK) or mouse antivimentin (Abcam) optimally diluted in TBS/BSA (1/200 and 2 µg/mL, respectively) was applied to slides for 60 min at room temperature. After being washed as above, for Oct-4 identification, fluorescein isothiocyanate-conjugated donkey polyclonal secondary antibody to goat IgG diluted in TBS/BSA in a ratio of 1:200 was used. For vimentin detection in Sertoli cells, Alexa Fluor 568-conjugated sheep antimouse IgG diluted in TBS/BSA in a ratio of 1:50 was added and incubation was further continued for 45 min at room temperature. After washing with TBS/ BSA, the nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Calbiochem, Nottingham, UK) at 0.1 µg/mL for 5 min, and then the slides were washed, mounted in PBS-glycerol 90%, and examined under a fluorescence microscope (Olympus). Primary antibodies were substituted by the same concentrations of the normal IgG1 in negative control slides and the results were shown to be always negative.

2.7. Statistical analysis

Results are presented as the mean ± SEM, and statistical analysis was performed by one-way analysis of variance and Duncan post hoc test. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Immunocytochemical staining of sorted cells

One sample from each calf was used in immunocytochemical analysis. Among the markers used for identification of SSCs, anti-Oct-4 was used. As expected, the cells of colonies were positive for these markers as shown in Figures 1a and 1b. The nuclei were counterstained by DAPI, as shown in the mentioned figures. Additionally, vimentin, which is a molecular marker for Sertoli cells, was detected in the feeder monolayer cells, which are shown in Figure 1c.

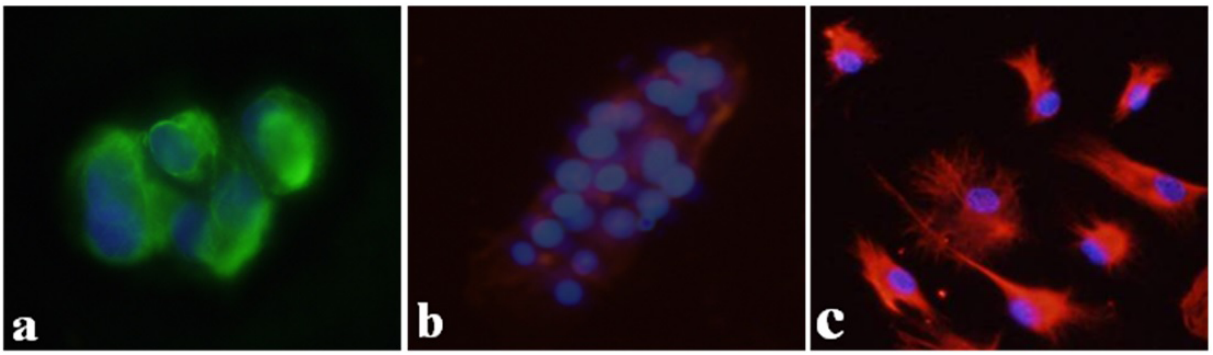

Figure 1. Immunocytochemical staining of SSCs and Sertoli cells. (a) Oct-4-positive colony. (b) Colonies were also positive for CD49f (a marker of Sertoli cells). (c) Vimentin was detected in the feeder monolayer cells (Sertoli cells). Nuclei were counterstained with DAPI. Magnification: a, 400×; b & c, 200×.

3.2. Colony assay in spermatogonial cells cocultured with Sertoli cells

The result of this study indicates that after 16 days of coculture, diameters of the colonies in groups 1 and 2 were significantly greater than in the other groups ($P < 0.05$), as shown in Figure 2. However, as shown in Figure 3, the number of colonies in group 2 ($P < 0.05$) was greater than in the control and group 1.

3.3. Viability rate after thawing

After 16 days of coculture, viability rates of the cells in the 4 groups were above 91%, but after the freezing process the viability rates between groups were different and the frozen-thawed cells' viability rate in group 2 ($P < 0.001$) was higher in comparison with other groups, as shown in Figure 4.

4. Discussion

Among the stem cells in the male body, only SSCs have the ability of transmitting genetic information to future generations (2). Thus, in cattle herds, SSCs are exploitable for genetic improvement (4). Large numbers of type A spermatogonia have been isolated and purified from young prepubertal bulls (4,5).

In this research, we isolated the cells of the seminiferous tubules of 3-month-old male calf testes, containing 2 types of cells: Sertoli cells and type A spermatogonia. From morphological aspects, the spermatogonial and Sertoli cells in our cultures were similar to the cells that Bellve et al. (17) expanded.

The size, morphology, and vimentin immunocytochemical staining were regarded as Sertoli

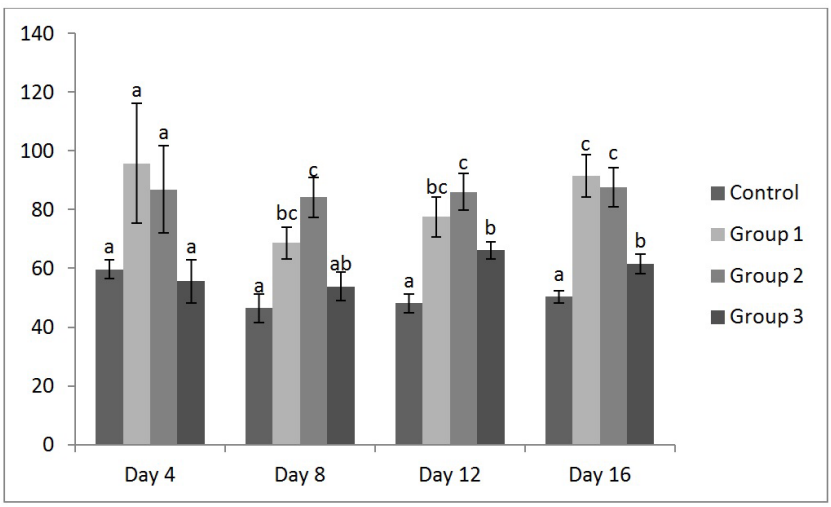

Figure 2. Comparison of colony diameters between control and experimental groups. Results from 5 separate experiments were used for all groups.
Different letters on columns for the same day represent significant differences ($P < 0.05$).

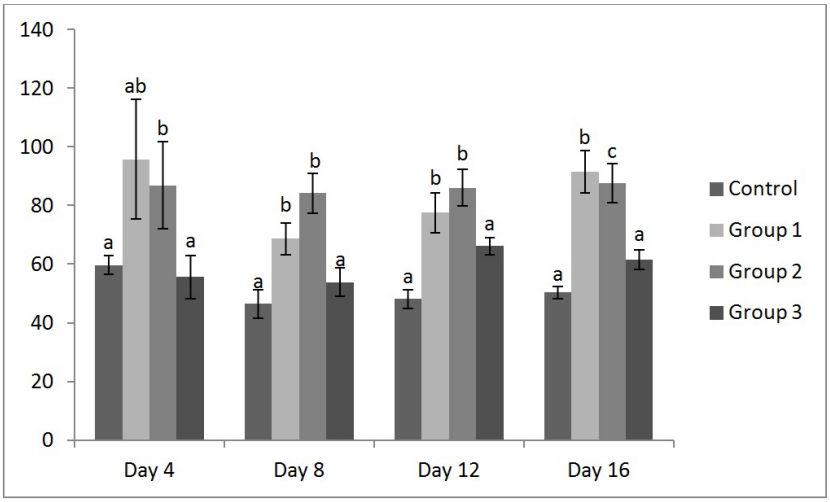

Figure 3. Comparison of colony numbers between control and experimental groups.
Results from 5 separate experiments were used for all groups.
Different letters on columns for the same day represent significant differences ($P < 0.05$).

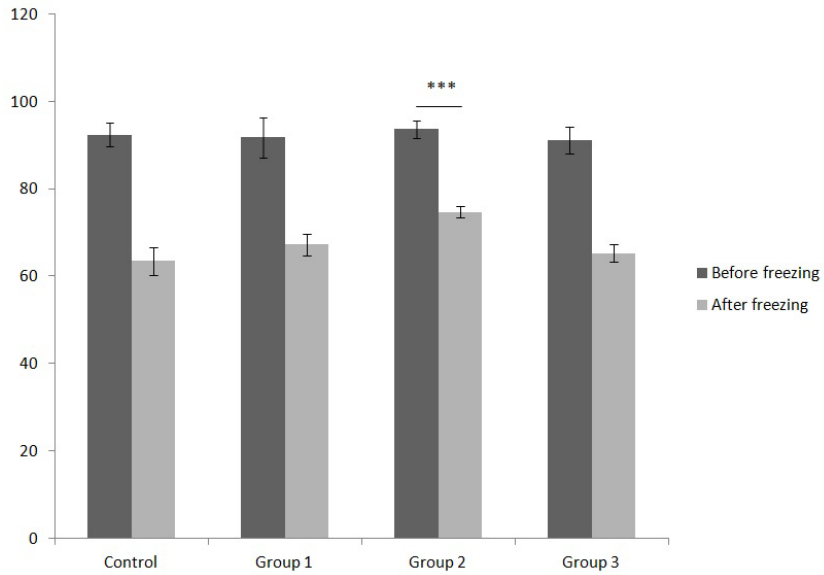

Figure 4. Comparison of viability rates between control and experimental groups before and after freezing.
Results from five separate experiments were used for all groups.
***: Significantly different at $P < 0.001$.

cell characterization parameters. Our results on expression of vimentin in bovine Sertoli cells were similar to the previous studies by Zimmerman et al. (18) and Tajik et al. (19). In addition to size and morphology, identification of spermatogonial cells was carried out by Oct-4 tracing in the colony cells. Spermatogonial-derived colonies showed Oct-4 expression. This finding is in agreement with reports of Kubota et al. (15), Jeong et al. (20), and Shi et al. (21), who verified Oct-4 expression in the SSCs.

Our method was a coculture of Sertoli and spermatogonia cells in vitro and we investigated the effects of IGF-1 on the colonization and viability of spermatogonia. After day 8 of coculture, the diameters of the colonies in groups 1 and 2 were larger than those of the control group, suggesting the proliferative effect of IGF-1. There were no significant differences between groups 1 and 2. Furthermore, the diameters of the colonies in groups 1 and 2 were larger than those of the group 3 after the 12th day.

The number of the colonies in groups 1 and 2 was higher than of the control and group 3. In the group 3, the number of the colonies did not show significant difference in comparison with the control group at the end of coculture.

Adding 40 or 100 ng/mL IGF-1 to the culture medium containing GDNF increased the number of SSCs at the 16th day of incubation. GDNF maintains proliferation and self-renewal of SSCs for years in vitro (22).The suggested mechanism was described by LeRoith et al. (23), where IGF-1 binds to its tyrosine kinase receptors and this triggers the phosphoinositide 3-OH kinase/Akt and mitogen-activated protein kinase pathways, which mediate cell proliferation or cell survival. Kubota et al. (15) also reported that the IGF system and GDNF signaling pathway may cooperate together in supporting self-renewal of SSCs.

In our study, the amount of 150 ng/mL IGF-1 had no positive effect on diameters and numbers of colonies. This is in agreement with Velazquez et al. (24), who stated that higher doses of IGF-1 induce downregulation of IGF-1 receptors.

The viability rate of frozen/thawed cells was lower than that of freshly isolated cells. This is mainly due to diminishing cell recovery following the freeze/thaw procedure. This finding was also reported by Avarbock et al. (25) and Brinster et al. (26). However, in group 2, the viability rate of frozen/thawed cells was higher than in other groups. Apparently, 100 ng/mL IGF-1 has an antiapoptotic effect on cells. Kennedy et al. (27), Khwaja et al. (28), and Kulik et al. (29) described IGF-1 receptors' antiapoptotic signaling pathway, which plays a role by phosphatidylinositol-3-kinase, the serine/threonine kinase Akt, and the MAP kinase downstream proteins.

In summary, these findings demonstrate that 100 ng/mL IGF-1 has a positive effect on SSC numbers and diameters. Furthermore, 100 ng/mL IGF-1 can increase frozen-thawed cell viability rate.

Acknowledgments

We are very grateful for the collaboration of the Avicenna Research Institute. In addition, we thank the Department of Animal Science of the University of Tabriz, Iran, and the Stem Cell Research Center of the Veterinary Faculty of the University of Tehran, Iran.

References

1. Zheng Y, Zhang Y, Qu R, He Y, Tian X, Zeng W. Spermatogonial stem cells from domestic animals: progress and prospects. Reproduction 2014; 147: R65–R74.
2. Dobrinski I. Germ cell transplantation and testis tissue xenografting in domestic animals. Anim Reprod Sci 2005; 89: 137–145.
3. Nagano M, Brinster CJ, Orwig KE, Ryu BY, Avarbock MR, Brinster RL. Transgenic mice produced by retroviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A 2001; 98: 13090–13095.
4. Aponte PM, Soda T, Teerds KJ, Mizrak SC, van de Kant HJG, de Rooij DG. Propagation of bovine spermatogonial stem cells in vitro. Reproduction 2008; 136: 543–557.
5. Aponte PM, Soda T, van de Kant HJG, de Rooij DG. Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. Theriogenology 2006; 65: 1828–1847.
6. Izadyar F, Den Ouden K, Creemers LB, Posthuma G, Parvinen M, De Rooij DG. Proliferation and differentiation of bovine type A spermatogonia during long-term culture. Biol Reprod 2003; 68: 272–281.
7. Izadyar F, Matthijs-Rijsenbilt JJ, Den Ouden K, Creemers LB, Woelders H, De Rooij DG. Development of a cryopreservation protocol for type A spermatogonia. J Androl 2002; 23: 537–545.
8. Koruji SM, Movahedin M, Mowla SJ, Gorabi H, Jabari Arfaei A. The effects of inducer factors on adult mouse spermatogonial cells colony formation in vitro. Yakhteh 2007; 9: 141–150.
9. Rinderknecht E, Humbel RE. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem 1978; 253: 2769–2776.
10. Blundell TL, Humbel RE. Hormone families: pancreatic hormones and homologous growth factors. Nature 1980; 287: 781–787.
11. Huang YH, Chin CC, Ho NN, Chou CK, Shen CN, Kuo HC, Wu TJ, Wu YC, Hung YC, Chang CC et al. Pluripotency of mouse spermatogonial stem cells maintained by IGF-1-dependent pathway. FASEB J 2009; 23: 2076–2087.
12. Bähr M, Kolter T, Seipke G, Eckel J. Growth promoting and metabolic activity of the human insulin analogue [Gly(A21),Arg(B31),Arg(B32)]insulin (HOE 901) in muscle cells. Eur J Pharmacol 1997; 320: 259–265.
13. Eckardt K, May C, Koenen M, Eckel J. IGF-1 receptor signalling determines the mitogenic potency of insulin analogues in human smooth muscle cells and fibroblasts. Diabetologia 2007; 50: 2534–2543.
14. Valentinis B, Morrione A, Peruzzi F, Prisco M, Reiss K, Baserga R. Anti-apoptotic signaling of the IGF-I receptor in fibroblasts following loss of matrix adhesion. Oncogene 1999; 18: 1827–1836.
15. Kubota H, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 2004; 101: 16489–16494.

16. Van Pelt AMM, Morena AR, Van Dissel-Emiliani FMF, Boitani C, Gaemers IC, De Rooij DG, Stefanini M. Isolation of the synchronized A spermatogonia from adult vitamin A- deficient rat testes. Biol Reprod 1996; 55: 439–444.

17. Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol 1977; 74: 68-85.

18. Zimmerman L, Lendahl U, Cunningham M, McKay R, Parr B, Gavin B, Mann J, Vassileva G, McMahon A. Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron 1994; 12: 11–24.

19. Tajik P, Barin A, Movahedin M, Zarnani AH, Hadavi R, Moghaddam G, Shoja J, Jeddi-Tehrani M, Ashrafi-Helan J, Heidari-Vala H et al. Nestin, a neuroectodermal stem cell marker, is expressed by bovine Sertoli cells. Comparative Clinical Pathology 2012; 21: 395–399.

20. Jeong D, McLean DJ, Griswold MD. Long-term culture and transplantation of murine testicular germ cells. J Androl 2003; 24: 661–669.

21. Shi YQ, Wang QZ, Liao SY, Zhang Y, Liu YX, Han CS. In vitro propagation of spermatogonial stem cells from KM mice. Front Biosci 2006; 11: 2614–2622.

22. Sariola H, Immonen T. GDNF maintains mouse spermatogonial stem cells in vivo and in vitro. Methods Mol Biol 2008; 450: 127–135.

23. LeRoith D, Roberts CT Jr. The insulin-like growth factor system and cancer. Cancer Lett 2003; 195: 127–137.

24. Velazquez MA, Zaraza J, Oropeza A, Webb R, Niemann H. The role of IGF1 in the in vivo production of bovine embryos from superovulated donors. Reproduction 2009; 137: 161–180.

25. Avarbock MR, Brinster CJ, Brinster RL. Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nat Med 1996; 2: 693–696.

26. Brinster RL, Nagano M. Spermatogonial stem cell transplantation, cryopreservation and culture. Semin Cell Dev Biol 1998; 9: 401–409.

27. Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, Tsichlis PN, Hay N. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev 1997; 11: 701–713.

28. Khwaja A, Rodriguez-Viciana P, Wennström S, Warne PH, Downward J. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J 1997; 16: 2783–2793.

29. Kulik G, Klippel A, Weber MJ. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol 1997; 17: 1595–1606.