



Biomaniipulation of bovine spermatogonial stem cells

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Abstract

The field of spermatogonial stem cell (SSC) technologies provides tools for genetic improvement of cattle herds and multiple opportunities for research. Spermatogonial stem cells belong to the male germ line and as such have high developmental potential, which offers many possibilities for transfer of relevant genetic traits across herds in a timely manner. Type A spermatogonia include a very small number of SSCs and their more numerous differentiating daughter cells. Initial attempts to isolate SSCs started with the isolation of type A spermatogonia and SSC purification. Type A spermatogonia can be obtained in large numbers from young prepubertal bulls, and it is important to note that there are breed differences. Type A spermatogonia isolation can be achieved through mechanical dissociation and enzymatic digestion of the testicular tissue followed by two purification steps, with a final typical bovine type A spermatogonia suspension of 70%. An evaluation for SSC activity using a transplantation assay adapted for bovine SSCs is described. Bovine Type A spermatogonia can be maintained *in vitro* for short periods (7 to 15 days) with simple culture conditions. However, expansion of SSC can only be achieved under certain conditions such as a specially supplemented medium, specific growth factors, and serial sub-culturing for longer periods of time. After expansion, bovine spermatogonia can be cryopreserved while retaining the ability to proliferate and survive. Despite all the challenges with development of SSC technologies, many questions arise focusing on how bovine SSCs work in a biotechnological setting. Progress in this field will probably result in new applications not only for bulls but also for other species with economical or ecological impact.

Keywords: biotechnology, bovine, germ cell transplantation, spermatogonial stem cells.

Introduction

The field of spermatogonial stem cell (SSC) technology has seen tremendous advances in recent years, mostly from research utilizing laboratory animals. Progress in cattle has been more modest, despite the

economical importance of the cattle industry, thus leaving opportunities for research and development of new technologies in the field. Spermatogonial stem cells belong to the male germ line, which include several families of undifferentiated cells with high developmental potential. These cells are biotechnologically important because they are the only cells in adult stem cell systems capable of transmitting genetic information to future generations (Dobrinski, 2006). This fact offers possibilities for SSC manipulation outside of the body in order to transfer relevant genes across herds. This is particularly important in cattle, which have a long generation interval (i.e., a nine months gestation period and 8 months birth to puberty period; Curtis and Amann, 1981) compared to laboratory animals and smaller species of livestock. A system in which genes could be delivered to cattle through SSC would considerably shorten the time to obtain transgenic animals. Thus, innovative SSC technologies for the transfer of genes to new generations will prove more effective and time-saving than other approaches such as the use of pluripotent embryonic stem cell manipulation or nuclear transfer (cloning; Dobrinski, 2006). Therefore, the development of techniques to isolate and culture SSC is becoming increasingly important.

Stem cells in general are able to divide to regenerate their own pool of cells (self-renewal) or alternatively produce daughter cells committed to differentiate (Fig. 1). They are important because they provide many proliferating cells in a transit amplifying compartment that will eventually produce large numbers of terminally differentiated cells with fully specialized capabilities (Alison *et al.*, 2002). During emergencies, such as when a deleterious agent disturbs the system in such a way that many transit amplifying and terminally differentiated cells die for instance, stem cells can repopulate the affected tissue in order to restore normal function.

There are several stem cell systems in the body in which stem cell activity controls the flow in the cellular line towards terminal differentiation. These systems include tissues as diverse as the skin, the digestive system lining, the hair follicle, bone marrow, and the seminiferous epithelium (Alison *et al.*, 2002). The stem cell system associated with the production of

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male gametes occurs within the testis, inside the seminiferous tubules, and it is called the seminiferous epithelium. The process of generation of sperm is called spermatogenesis, and the stem cells in this system in the adult are the SSCs. These cells lie at the base of the seminiferous epithelium and coexist with their differentiating daughter cells in a space called the basal compartment, which is created by the surrounding Sertoli cells (Russell *et al.*, 1990). Proliferating cells committed to differentiate, which are located in that space, represent the transit amplifying compartment in

the spermatogenic process. When harmful agents (i.e., chemicals, radiation, etc.) destroy the more sensitive differentiating germ cells, SSCs are able to self-renew at first and then gradually repopulate the seminiferous tubule to restore spermatogenesis (de Rooij and Russell, 2000). Stem cells in general require a special microenvironment in which signal networks and nutrients converge to induce them to maintain the undifferentiated state, a place called niche (Fuchs *et al.*, 2004; Li and Xie, 2005). The niche for SSCs is provided by Sertoli cells (Simon *et al.*, 2007).

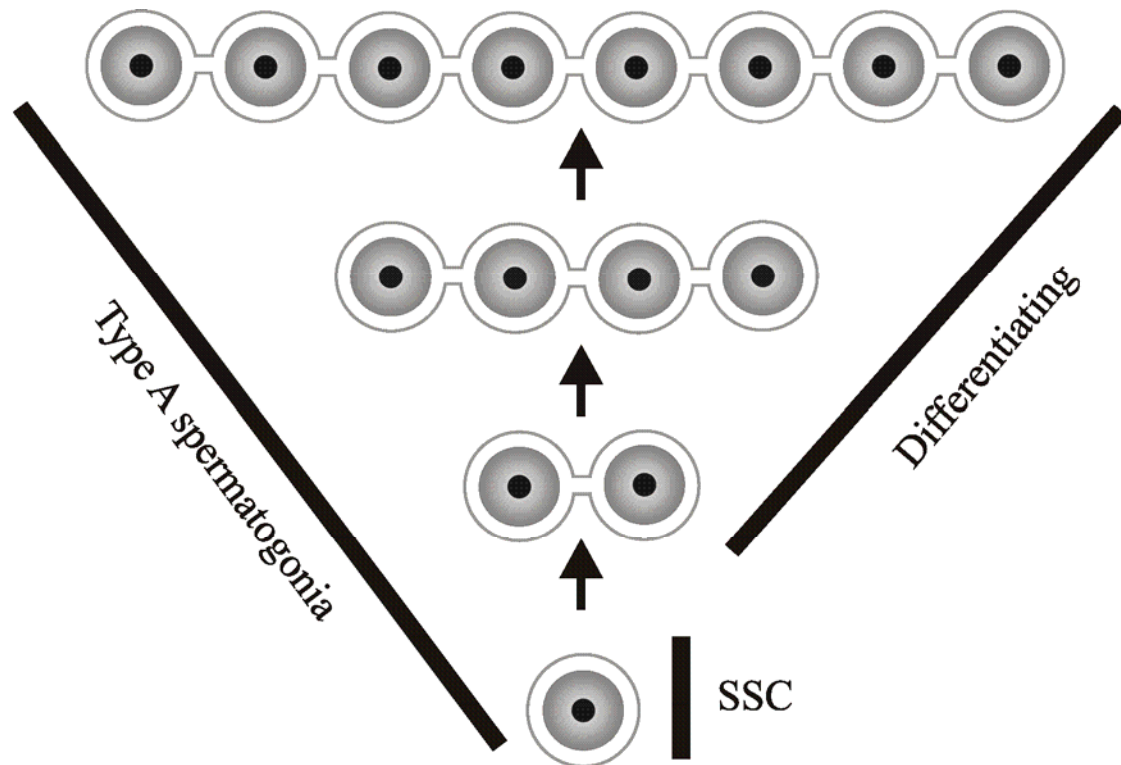


Figure 1. Diagram of the first divisions of spermatogonial stem cells (SSCs). These cells divide to either produce SSC daughter cells (self-renewal) or differentiating daughter cells. Type A spermatogonia committed to differentiate remain connected by intercellular bridges after division. For the rest, SSCs and their differentiating daughters, both type A spermatogonia are morphologically undistinguishable.

Spermatogonial stem cells and their initial differentiating daughter cells are extremely difficult to distinguish from a morphological point of view. Both cell types constitute a group of cells collectively called type A spermatogonia. Therefore, SSCs represent a subpopulation of the type A spermatogonia group (Fig. 1). During the formation of differentiating type A spermatogonia from a mother SSC, cytokinesis is not complete so that differentiating type A spermatogonia remain interconnected by intercellular bridges. In fact, in the adult testis, the first visible sign of the choice of SSC towards a differentiation fate is the formation of two daughter cells interconnected by an intercellular bridge (de Rooij and Russell, 2000).

Initial attempts of isolating SSCs started with the isolation of type A spermatogonia as a first step with the intention to further purify them to obtain the subpopulation of stem cells. In cattle, type A spermatogonia are round- to oval-shaped cells with a high nucleus:cytoplasm ratio and usually a large central nucleolus (Abdel-Raouf, 1960; Curtis and Amann, 1981). Type A spermatogonia can be obtained in fairly large quantities from young prepubertal bulls. It is important to take into account the time of the start of spermatogenesis, since this is the time when gonocytes start to divide to produce type A spermatogonia, which marks the beginning of the prepubertal period (Curtis and Amann, 1981). Testes should be collected from animals shortly after the start of

spermatogenesis and before spermatocytogenesis starts. In *Bos taurus* (European breeds) cattle, this occurs at 4 to 5 months of age, while in *Bos indicus* cattle (i.e., Brahman breed bulls, of Asian origin) this window corresponds to 10 to 11 months of age (Aponte *et al.*, 2005). Isolation from older animals will yield lower concentrations of type A spermatogonia in suspension since these cells will be diluted in a milieu composed of all other more advanced germ cells (i.e., spermatocytes, spermatids and sperm), which appear gradually as the testes develop towards the pubertal stage.

Type A spermatogonia isolation procedure

Type A spermatogonia isolation can be achieved using a two-day protocol, first performing a mechanical dissociation followed by an enzymatic digestion of the testicular tissue (Izadyar *et al.*, 2002b). Testes are kept on ice after castration or retrieval from

slaughterhouse animals for a maximum of 4 hours with no apparent loss of spermatogonia viability. Because of the size of the bovine testis, a sample of as much as 20 grams of testicular parenchyma can be managed during each isolation procedure. After enzymatic treatments, a differential plating procedure will eliminate most somatic cells, as they tend to attach to the bottom of a culture flask (Fig. 2). This leaves a cell suspension of about 40% pure bovine type A spermatogonia recovered at the start of the second day. A higher level of purity is achieved by using a Percoll discontinuous density gradient (van Pelt *et al.*, 1996; Izadyar *et al.*, 2002b). Bovine type A spermatogonia will usually be located in Fraction 2 or 3 (Fig. 2). A typical final cell suspension will have 70% of type A bovine spermatogonia with the other 30% made of contaminant somatic cells, mainly Sertoli cells (Aponte *et al.*, 2006), myoid peritubular cells, and a negligible amount of Leydig cells (unpublished data; Aponte *et al.*, 2008).

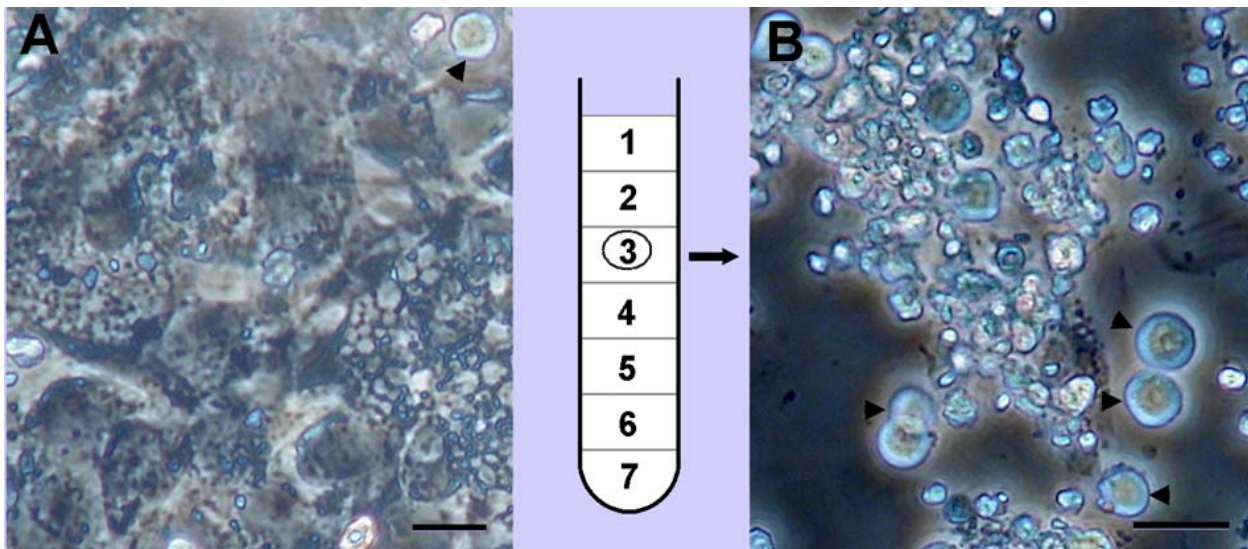


Figure 2. Some steps of type A spermatogonial purification: A) Culture flask bottom after differential plating and very few type A spermatogonia (arrowhead) stay atop the somatic cells and B) Type A spermatogonia after discontinuous density gradient. Fraction 3 usually contains the most bovine type A spermatogonia (arrowheads).

Bovine SSC identity

Further straining of type A spermatogonia cell suspensions to obtain highly pure SSC has not been yet successfully performed in bovine species. This could be achieved through the development of bovine specific antibodies and the use of fluorescent-activated cell sorting (FACS; Shinohara *et al.*, 2000; Ryu *et al.*, 2004) or magnetic activated cell sorting (MACS; von Schonfeldt *et al.*, 1999; van der Wee *et al.*, 2001; Kubota *et al.*, 2004a,b) in adaptation to this species. Ultimately, alleged SSC-containing, type A spermatogonial suspensions can be tested for stem cell activity through transplantation techniques. Such

procedures, originally developed to evaluate mouse spermatogonial stem cell activity, can be readily used with cattle (Izadyar *et al.*, 2003; Aponte *et al.*, 2006). With this technique, a cell population can be assessed for the presence of spermatogonial stem cells, thus allowing for the development of purification protocols. In this procedure, donor germ cells (i.e., bovine spermatogonia) are transplanted into the testis of a recipient mouse, in which spermatogenesis has been depleted either because of a Wv/Wv mutation or by treatment with an alkylating agent, busulfan. An alternative method is to deplete spermatogenesis in the recipient mice using fractionated X-irradiation (local testicular doses of 1.5 and 12 Gy, 24 hours apart). This

protocol reliably causes more than 95% depletion (Creemers *et al.*, 2002b). After transplantation, the donor spermatogonial stem cells repopulate the seminiferous epithelium of the recipient mice. Interestingly, in a model of phylogenetically close species like the mouse and rat, rat SSCs can repopulate a mouse testis and reproduce normal rat spermatogenesis (Clouthier *et al.*, 1996; Russell and Brinster, 1996). However, stem cells from non-related species transplanted into the mouse testis are able to initiate the repopulation but the differentiating daughter cells will stop proliferating at some point with no further development, as in rabbits and dogs (Dobrinski *et al.*, 1999), bulls (Izadyar *et al.*, 2003), and baboons (Nagano *et al.*, 2001). Although transplanted bovine type A spermatogonia will not proliferate to produce spermatogenetic colonies that can be considered originating from one SSC, as in the mouse to mouse transplantation model (Kanatsu-Shinohara *et al.*, 2006), they are considered SSCs for two reasons: a) they find their way to the basal compartment of the mouse seminiferous epithelium (SSC niche), a process called homing (Nagano, 2003) and b) they are still in that location after two months or longer, as this period of time corresponds to the duration of spermatogenesis in bulls (61 days), that is, the time needed for the whole process of a stem cell to produce sperm in the bull (Amann, 1983). Therefore, any original non-germ stem cell “contaminant” will have enough time to undergo the whole process and finally be shed as sperm.

After two months, the transplanted mice are killed and the testes recovered to identify the SSC with markers. Many specific SSC markers for small experimental animals (i.e. mouse and rat) are not adaptable for use with the bovine species. Fortunately, we have repeatedly and successfully used byotinated DBA lectin in order to identify bovine type A spermatogonia (Izadyar *et al.*, 2002a,b, 2003; Aponte *et al.*, 2006). The use of this marker is originally based on an extensive lectin characterization of the bovine testis (Ertl and Wrobel, 1992). This staining procedure is a reliable and simple and is a one-day protocol. Another alternative is the use of PKH26 (a general fluorescence membrane marker; Oatley *et al.*, 2004; Herrid *et al.*, 2006) that bypasses the problem of obtaining bovine specific antibodies. More research is required to find additional specific markers for bovine SSCs. Similar to the mouse (Shinohara *et al.*, 1999; Kubota *et al.*, 2003), bovine SSCs likely have a phenotypic profile composed of several proteins, which is more realistic and accurate than relying on a unique paramount marker for characterization purposes.

Bovine DBA-positive cells in histological sections from transplanted mouse testes will be found as single cells most of the time. They can be counted in an unbiased way by using stereological methods. The appropriate stereological method for unbiased counting of particles like SSCs is using “the Optical disector”

(Howard and Reed, 1998). Alternatively, a much simpler and less time-consuming *in vitro* assay for evaluating bovine SSC activity could be developed as was recently validated in the mouse (Yeh *et al.*, 2007).

Basic culture

Bovine type A spermatogonia can be maintained *in vitro* for short periods of time (7 to 15 days) with simple culture conditions. In general, these conditions include the use of a basic medium (minimal essential medium, MEM) supplemented with NaHCO₃, L-glutamine, non-essential amino acids, antibiotics (penicillin–streptomycin, gentamicin), Hepes buffer, and serum (fetal calf serum, FCS) at a concentration of 2.5%, an incubation temperature of 37°C, atmosphere of 5% CO₂ in a humid chamber, and seeding densities of 100 cells/μl for cell suspensions (Izadyar *et al.*, 2003; Aponte *et al.*, 2006). Additionally, bovine culture systems require some form of feeder layer to support the germ cells. Autogenous Sertoli cells (product of “contamination” during the purification process) (Aponte *et al.*, 2006) or embryonic fibroblasts (Oatley *et al.*, 2004) have been used.

Use of serum

It is not yet clear whether or not serum in the culture has positive or negative effects on bovine spermatogonial stem cells. One of the most common arguments against the use of serum is the presence of unknown and variable factors that may hinder the interpretation of the results (Kubota *et al.*, 2004a). In bovine cultures, at least 1% FCS should be used because cells do not maintain well in culture with lower levels. This fact has been reported for the mouse (Kanatsu-Shinohara *et al.*, 2005) while other laboratories have introduced the use of serum-free culture systems and still obtained enhanced stem cell renewal (Kubota *et al.*, 2004b; Kanatsu-Shinohara *et al.*, 2005). Nevertheless, the aspect of usage of serum in bovine spermatogonial systems is controversial and should receive more research efforts.

Use of feeder layers

The advantages of using co-cultures for the maintenance of spermatogonial stem cells *in vitro* are related to the specific cell type or line used. It has been suggested that somatic cell contamination (Creemers *et al.*, 2002a) or the use of Sertoli-cell-based monolayers (Nagano *et al.*, 2003) seem to halt the self-renewal process of spermatogonial stem cells. Differentiation (Nagano *et al.*, 2003) or self-renewal inhibition signals produced and secreted by Sertoli cells like inhibin, as suggested by clonal culture of gonocytes and spermatogonia (Hasthorpe, 2003), have been proposed as mechanisms for this negative effect. However, as



SSCs require a niche provided by Sertoli cells *in vivo*, probably this microenvironment can be reproduced *in vitro*. We hold this as valid for the bovine culture system. Accordingly, a good system would be that composed of a starting population of a highly purified SSC suspension with autogenous Sertoli cells. These epithelial cells provide self-renewal proteins to SSCs in their niches in the testis (Meng *et al.*, 2000; Simon *et al.*, 2007), but the production of these proteins in bovine *in vitro* systems remains to be verified. Although the use of Sertoli cell feeder layers is a controversial issue, it encourages further research.

SSC propagation in culture

Stem cell numbers in culture unfortunately tend to decline with time; therefore, at best maintenance is guaranteed but not expansion. In the mouse, progress regarding this aspect was achieved by applying additional factors favorable for SSC renewal (Kanatsu-Shinohara *et al.*, 2003a; Kubota *et al.*, 2004a,b). Kanatsu-Shinohara *et al.* (2003a) introduced an improved culture system based on a commercial medium (StemPro-34® SFM, Invitrogen) supplemented with various agents, hormones, and growth factors including β -estradiol, progesterone, Epidermal Growth Factor (EGF), basic Fibroblast Growth Factor (bFGF), Leukemia Inhibitory Factor (LIF), and Glial-cell line-derived Neurotrophic Factor (GDNF). Only when these conditions were met did SSC proliferate and form colonies during the first week of culture. This approach included continuous sub-culturing for 4 to 5 months, thus achieving an expansion of spermatogonial stem cells in the order of 10^{12} fold. This culture system works effectively with bovine species, which is evident by the more than 14000-fold increase in the number of SSCs after 30 days of culture (unpublished data; Aponte *et al.*, 2008).

Cryopreservation

Once obtained, bovine spermatogonia can be cryopreserved. Frozen-thawed bovine SSCs are able to survive the cryopreservation process (Izadyar *et al.*, 2002a; Oatley *et al.*, 2004). The use of a freezing MEM-based medium containing 10% FCS, 10% DMSO, and 0.07M sucrose and a noncontrolled-rate freezing protocol is an effective way to preserve type A spermatogonia, with a concentration of living cells after thawing of about 70%. Additionally, it has been suggested that SSCs survive the freeze-thaw process better than other cell types in the mouse (Kanatsu-Shinohara *et al.*, 2003b). This preferential survival ratio remains to be tested in the bovine species. In general, frozen/thawed spermatogonia survive in culture and retain the ability to proliferate (Izadyar *et al.*, 2002a; Oatley *et al.*, 2004).

Final remarks

Despite all the challenges that the development of SSC technologies raise, still many questions are open as to how bovine SSCs work in a biotechnological setting. Progress in the field will probably derive new applications not only for use in bulls but also in related large-animal species like water buffaloes and other less-studied bovine subspecies like *Bos indicus*, which are of great importance to the food production chains of many developing countries.

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References

- Abdel-Raouf M.** 1960. The postnatal development of the reproductive, organs in bulls with special reference to puberty including growth of the hypophysis and the adrenals. *Acta Endocrinol (Journal)*, 34(suppl 49):1-109.
- Alison MR, Poulosom R, Forbes S, Wright NA.** 2002. An introduction to stem cells. *J Pathol*, 197:419-423.
- Amann RP.** 1983. Endocrine changes associated with onset of spermatogenesis in Holstein bulls. *J Dairy Sci*, 66:2606-2622.
- Aponte PM, de Rooij DG, Bastidas P.** 2005. Testicular development in Brahman bulls. *Theriogenology*, 64:1440-1455.
- Aponte PM, Soda T, van de Kant HJ, de Rooij DG.** 2006. Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. *Theriogenology*, 65:1828-1847.
- Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL.** 1996. Rat spermatogenesis in mouse testis. *Nature*, 381:418-421.
- Creemers LB, den Ouden K, van Pelt AM, de Rooij DG.** 2002a. Maintenance of adult mouse type A spermatogonia *in vitro*: influence of serum and growth factors and comparison with prepubertal spermatogonial cell culture. *Reproduction*, 124:791-799.
- Creemers LB, Meng X, den Ouden K, van Pelt AM, Izadyar F, Santoro M, Sariola H, de Rooij DG.** 2002b. Transplantation of germ cells from glial cell line-derived neurotrophic factor-overexpressing mice to host testes depleted of endogenous spermatogenesis by fractionated irradiation. *Biol Reprod*, 66:1579-1584.
- Curtis SK, Amann RP.** 1981. Testicular development and establishment of spermatogenesis in Holstein bulls. *J Anim Sci*, 53:1645-1657.



- de Rooij DG, Russell LD.** 2000. All you wanted to know about spermatogonia but were afraid to ask. *J Androl*, 21:776-798.
- Dobrinski I, Avarbock MR, Brinster RL.** 1999. Transplantation of germ cells from rabbits and dogs into mouse testes. *Biol Reprod*, 61:1331-1339.
- Dobrinski I.** 2006. Transplantation of germ cells and testis tissue to study mammalian spermatogenesis. *Anim Reprod*, 3:135-145.
- Ertl C, Wrobel KH.** 1992. Distribution of sugar residues in the bovine testis during postnatal ontogenesis demonstrated with lectin-horseradish peroxidase conjugates. *Histochemistry*, 97:161-171.
- Fuchs E, Tumber T, Guasch G.** 2004. Socializing with the neighbors: stem cells and their niche. *Cell*, 116:769-778.
- Hasthorpe S.** 2003. Clonogenic culture of normal spermatogonia: *in vitro* regulation of postnatal germ cell proliferation. *Biol Reprod*, 68:1354-1360.
- Herrid M, Vignarajan S, Davey R, Dobrinski I, Hill JR.** 2006. Successful transplantation of bovine testicular cells to heterologous recipients. *Reproduction*, 1324:617-624.
- Howard CV, Reed MG.** 1998. *Unbiased Stereology: Three-dimensional Measurement in Microscopy*. London: BIOS Scientific Publishers Limited.
- Izadyar F, Matthijs-Rijsenbilt JJ, den Ouden K, Creemers LB, Woelders H, de Rooij DG.** 2002a. Development of a cryopreservation protocol for type A spermatogonia. *J Androl*, 23:537-545.
- Izadyar F, Spierenberg GT, Creemers LB, den Ouden K, de Rooij DG.** 2002b. Isolation and purification of type A spermatogonia from the bovine testis. *Reproduction*, 124:85-94.
- Izadyar F, den Ouden K, Creemers LB, Posthuma G, Parvinen M, de Rooij DG.** 2003. Proliferation and differentiation of bovine type A spermatogonia during long-term culture. *Biol Reprod*, 68:272-281.
- Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T.** 2003a. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod*, 69:612-616.
- Kanatsu-Shinohara M, Ogonuki N, Inoue K, Ogura A, Toyokuni S, Shinohara T.** 2003b. Restoration of fertility in infertile mice by transplantation of cryopreserved male germline stem cells. *Hum Reprod*, 18:2660-2667.
- Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T.** 2005. Long-term culture of mouse male germline stem cells under serum- or feeder-free conditions. *Biol Reprod*, 724:985-991.
- Kanatsu-Shinohara M, Inoue K, Miki H, Ogonuki N, Takehashi M, Morimoto T, Ogura A, Shinohara T.** 2006. Clonal origin of germ cell colonies after spermatogonial transplantation in mice. *Biol Reprod*, 75:68-74.
- Kubota H, Avarbock MR, Brinster RL.** 2003. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proc Natl Acad Sci USA*, 100:6487-6492.
- Kubota H, Avarbock MR, Brinster RL.** 2004a. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. *Biol Reprod*, 71:722-731.
- Kubota H, Avarbock MR, Brinster RL.** 2004b. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci USA*, 101:16489-16494.
- Li L, Xie T.** 2005. Stem cell niche: structure and function. *Annu Rev Cell Dev Biol*, 21:605-631.
- Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H.** 2000. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*, 287:1489-1493.
- Nagano M, McCarrey JR, Brinster RL.** 2001. Primate spermatogonial stem cells colonize mouse testes. *Biol Reprod*, 64:1409-1416.
- Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL.** 2003. Maintenance of mouse male germ line stem cells *in vitro*. *Biol Reprod*, 68:2207-2214.
- Nagano MC.** 2003. Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. *Biol Reprod*, 69:701-707.
- Oatley JM, Reeves JJ, McLean DJ.** 2004. Biological activity of cryopreserved bovine spermatogonial stem cells during *in vitro* culture. *Biol Reprod*, 71:942-947.
- Russell LD, Ettl RA, Sinha-Hikim AP, Clegg ED.** 1990. *Histological and Histopathological Evaluation of the Testis*. Clearwater, FL: Cache River Press.
- Russell LD, Brinster RL.** 1996. Ultrastructural observations of spermatogenesis following transplantation of rat testis cells into mouse seminiferous tubules. *J Androl*, 17:615-627.
- Ryu BY, Orwig KE, Kubota H, Avarbock MR, Brinster RL.** 2004. Phenotypic and functional characteristics of spermatogonial stem cells in rats. *Dev Biol*, 274:158-170.
- Shinohara T, Avarbock MR, Brinster RL.** 1999. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. *Proc Natl Acad Sci USA*, 96:5504-5509.
- Shinohara T, Orwig KE, Avarbock MR, Brinster RL.** 2000. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *Proc Natl Acad Sci USA*, 97:8346-8351.
- Simon L, Ekman GC, Tyagi G, Hess RA, Murphy KM, Cooke PS.** 2007. Common and distinct factors regulate expression of mRNA for ETV5 and GDNF, Sertoli cell proteins essential for spermatogonial stem cell maintenance. *Exp Cell Res*, 313:3090-3099.
- van der Wee KS, Johnson EW, Dirami G, Dym TM, Hofmann MC.** 2001. Immunomagnetic isolation and long-term culture of mouse type A spermatogonia. *J*



Androl, 22:696-704.

van Pelt AM, Morena AR, van Dissel-Emiliani FM, Boitani C, Gaemers IC, de Rooij DG, Stefanini M.

1996. Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. *Biol Reprod*, 55:439-444.

von Schonfeldt V, Krishnamurthy H, Foppiani L,

Schlatt S. 1999. Magnetic cell sorting is a fast and effective method of enriching viable spermatogonia from Djungarian hamster, mouse, and marmoset monkey testes. *Biol Reprod*, 61:582-589.

Yeh JR, Zhang X, Nagano MC. 2007. Establishment of a short-term *in vitro* assay for mouse spermatogonial stem cells. *Biol Reprod*, 77:897-904.
