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REVIEW

Developments in techniques for the isolation, enrichment, main culture conditions and identification of spermatogonial stem cells

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Abstract The in vitro culture system of spermatogonial stem cells (SSCs) provides a basis for studies on spermatogenesis, and also contributes to the development of new methods for the preservation of livestock and animal genetic modification. In vitro culture systems have mainly been established for mouse SSCs, but are lacking for farm animals. We reviewed and analyzed the current progress in SSC techniques such as isolation, purification, cultivation and identification. Based on the published studies, we concluded that two-step enzyme digestion and magnetic-activated cell sorting are fast becoming the main methods for isolation and enrichment of SSCs. With regard to the culture systems, serum and feeders were earlier thought to play an important role in the self-renewal and proliferation of SSCs, but serum- and feeder-free culture systems as a means of overcoming the limitations of SSC differentiation in long-term SSC culture are being explored. However, there is still a need to establish more efficient and ideal culture systems that can also be used for SSC culture in larger mammals. Although the lack of SSC-specific surface markers has seriously affected the efficiency of purification and identification, the transgenic study is helpful for our identification of SSCs. Therefore, future studies on SSC techniques should focus on improving serum- and feeder-free culture techniques, and discovering and identifying specific surface markers of SSCs, which will provide new ideas for the optimization of SSC culture systems for mice and promote related studies in farm animals.

Keywords Spermatogonial stem cells · Surface marker · Purification · Cultivation

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Introduction

Spermatogenesis is an efficient and well-organized process that occurs within the seminiferous tubules of the mammalian testis through a complex mechanism (Nagano et al. 1998). Spermatogonial stem cells (SSCs) represent a small percentage of germ line cells, and play an important role in the self-renewal, continuous proliferation, and differentiation of sperm cells, and therefore in the maintenance of spermatogenesis (Lee et al. 2007). These biological functions make SSCs extensively useful in the field of biology, medicine, genetics and gene engineering. High-efficiency SSC culture systems are of significant importance in the animal husbandry industry and even in the treatment of human diseases; moreover, SSC culture

has also opened up new effective methods for animal genetic modification. A number of investigations have been performed on the mouse SSC culture system (Guan et al. 2009; Kanatsu-Shinohara et al. 2003; Nagano et al. 1998). However, the results of SSC cultures vary among culture systems, animal species, and even different experimental repetitions (Kanatsu-Shinohara et al. 2003; van der Wee et al. 2001), and the SSC culture system in large animals is still being explored (Aponte et al. 2008; Gautam et al. 2012). Moreover, although some methods for SSC isolation and purification have been established, their purification efficiency is poor. The reason for this study bottleneck in SSC purification and culture is the unclear differentiation mechanism and lack of SSC-specific markers (Kanatsu-Shinohara et al. 2012a). Therefore, there is a need to summarize and analyze the breakthroughs in SSC isolation, purification, cultivation, and identification techniques, because this can help future studies establish a stable and sound cultivation and identification system for SSCs, and would also be useful for SSC research in large mammals.

SSC isolation

The seminiferous epithelium contains only primitive type A spermatogonia (SSCs) and Sertoli cells in the day 6 mouse, after which type B spermatogonia, primary spermatocytes, secondary spermatocytes and haploid spermatids gradually emerge (Bellve et al. 1977; Zou et al. 2009), so the proportion of SSCs gradually decreases (Fig. 1): in the adult mouse testis, only 3.5×10^4 of 10^8 germ cells are thought to be SSCs (Kanatsu-Shinohara et al. 2013; Tegelenbosch and de Rooij 1993). Since cyptorchidism can suppress SSC differentiation and spermatogenesis (little or no c-kit expression is found in cell suspension from adult cryptorchid testes), cryptorchid operation is often used to enrich the SSC population; it results in an approximately 20- to 25-fold (about 1 in 200 cells is an SSC) enrichment of SSCs compared to wild-type testes (Kubota et al. 2004a; Shinohara et al. 2000a, b). Although cryptorchid operation provides a highly efficient approach for SSC isolation, it is a time-consuming and highly invasive method, so it is not applicable to several animal species (Aponte et al. 2008; Kanatsu-Shinohara et al. 2012b). Another technique for SSC isolation involves mechanical disruption combined with trypsin dispersion of seminiferous tubules; in this method, the seminiferous tubules are cut into very small fragments by scissors and then incubated in trypsin. However, SSCs can get seriously damaged and washed away by this method. To avoid these shortcomings, the tunica albuginea was dissected and removed mechanically from the testis first, and it was then dissociated via a two-step enzymatic incubation process (Bellve et al. 1977; Gautam et al. 2012; Izadyar et al. 2002; Kossack et al. 2009; Ogawa et al. 1997). In brief, seminiferous tubules were dispersed with collagenase, and then the seminiferous tubule fragments were incubated in trypsin to generate a single cell suspension (Brinster and Avarbock 1994). In order to obtain a high-quality suspension, researchers combine hyaluronidase and DNase I (two-step enzymatic process), via which cells are isolated through digestion of cell–cell junctions and genomic DNA is digested to reduce the viscosity of the cell mixture (Guan et al. 2009; Kofman et al. 2013). The two-step enzyme digestion process has high reproducibility, and therefore this economical and simple technique has become the main method for SSC isolation.

SSC purification

Glial cell line-derived neurotrophic factor (GDNF) was proposed to be an important factor for communication between SSCs and Sertoli cells (SCs) (Kanatsu-Shinohara et al. 2005a). SCs provide the microenvironment and secrete GDNF for SSCs (Yomogida et al. 2003). GDNF promotes the proliferation and inhibits the differentiation of SSCs; however, SSCs eventually disappear owing to the extensive growth of SCs (Kanatsu-Shinohara et al. 2005a). The paradoxical relationship between extensive growth of SCs and proliferation of SSCs indicates that it is an important factor to be considered for establishing a long-term culture system for SSCs and obtaining high-purity SSCs. Therefore, a series of SSC purification methods have been established, such as differential plating selection, gravity sedimentation selection, discontinuous percoll density gradient centrifugation selection, morphology-based selection, laminin selection, fluorescence-activated cell sorting (FACS)

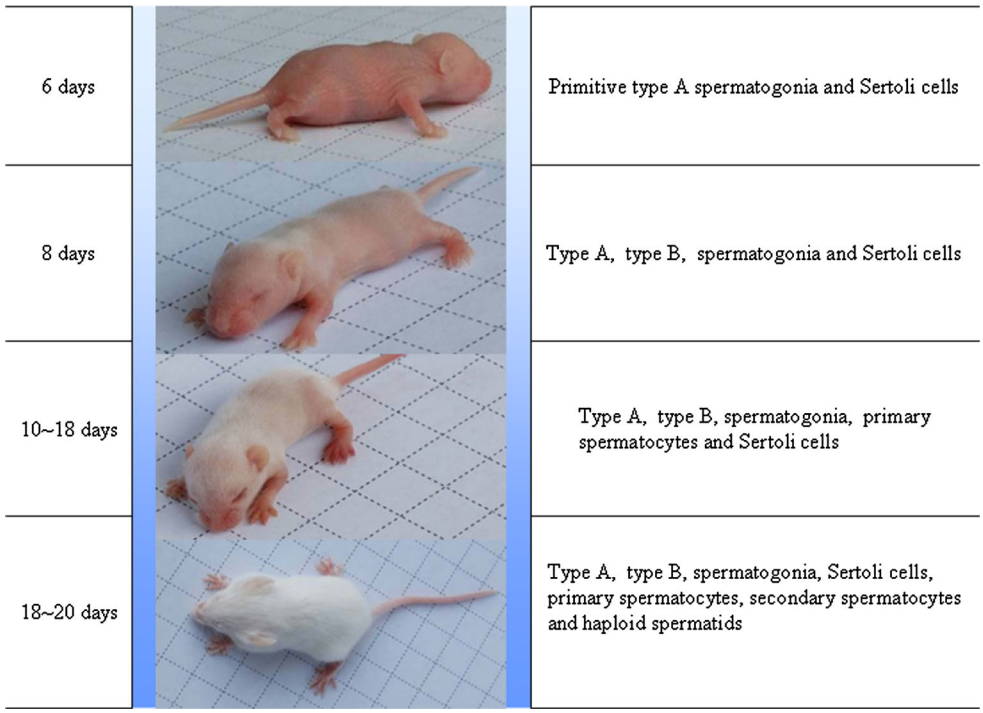

Fig. 1 The development periods and the correspond germ line cell types in postnatal mouse testes. The seminiferous epithelium from day 6 contains only primitive type A spermatogonia and Sertoli cells. Type B spermatogonia appear by day 8. At day 10, meiotic prophase is initiated, with the germ cells reaching the early and late pachytene stages by days 14 and 18, respectively. Secondary spermatocytes and haploid spermatids appear in increasing numbers between days 18 and 20 (Bellve et al. 1977)

selection, and magnetic-activated cell sorting (MACS) selection.

Physical methods for SSC purification

As SCs adhere to the plate faster than SSCs, differential plating selection, based on the differential adherence speed between SCs and SSCs, can eliminate a part of high-activity SCs. However, if the adherence time is not judged correctly or if the operator lacks experience, a large amount of adherent SSCs with high activity may also be lost. Based on the differential settling velocity, gravity sedimentation selection on a 2–4 % bovine serum albumin (BSA) gradient is also used for SSC purification (Bellve et al. 1977; Hofmann et al. 2005). Based on a similar principle, discontinuous percoll density gradient centrifugation selection is another technique used for SSC purification. In this method, the SSC suspension is used as the top layer of a discontinuous Percoll concentration gradient and then centrifuged; at the end of the process, the SSCs are retained in a suitable Percoll gradient fraction (Izadyar et al. 2002). However, the various cell layers are not obvious, and the amount of time the method requires distinctly affects SSC activity. Morphology-based selection is another SSC purification method that is simple and economical. However, SCs show more growth than SSCs at the early stages of derivation, so it is necessary to mechanically isolate SSCs colonies under a microscope and passage them to a fresh feeder culture plate as soon as they appear, this requires the use of an efficient technique or even a combination of methods (Guan et al. 2009).

Physical methods are based on the biological characteristics of SSCs and allow for further purification of SSCs; the purification efficiency, however, is relatively low. To improve the purification efficiency, a series of studies were undertaken, and some stem cell markers expressed on SSCs were found. For example, the surface markers β1-and α6-integrin were found on mouse SSCs (Shinohara et al. 1999). These molecules comprise a known receptor for the extracellular matrix

component laminin (Guan et al. 2009); transplantation experiments based on this ligand-receptor relationship showed that laminin-bound cells colonized recipient testes three to fourfold better than control cells (Shinohara et al. 1999). Therefore, exploring surface markers and developing new purification methods are already becoming the focus in this field.

Immunological methods for SSC purification

Based on the specific conjugation between surface markers and their monoclonal or polyclonal antibodies (or ligands), FACS, an immunological SSC purification method, was established. Stra8 is a spermatogonial-specific marker, and its promoter was used to express the fluorescent marker gene enhanced green fluorescent protein (EGFP) in Stra8–EGFP/Rosa26 transgenic mice. The activity of the regulatory sequence of Stra8 enables the enrichment of SSCs from transgenic mice (Giuili et al. 2002). Testicular cells were isolated from Stra8–EGFP/Rosa26 adult mice and cultured for 4–7 days, and then GFP^+ cells were isolated using FACS at as high a rate as 27 % (fivefold enrichment compared with the control group); then, the isolated cells were transplanted into recipient mice, and regeneration of normal spermatogenesis was observed (Guan et al. 2006). When multiple antibodies are used simultaneously, FACS can sort SSCs more rapidly and specifically. Therefore, multi-parameter flow cytometry was developed based on single-antibody immunophenotyping (Stewart 2000). The experiments showed that SSCs express melanoma cell adhesion molecule (MCAM), which belongs to the immunoglobulin superfamily and mediates cation-independent adhesion. Multiparameter flow cytometry selection of adult testis cells with a $CD9^+EPCAM^{low}MCAM^+KIT^-$ phenotype resulted in a 561-fold enrichment of SSCs (Kanatsu-Shinohara et al. 2012b). Even though FACS has the most desirable outcome, it requires the use of some expensive technology and relies on large proprietary equipment, which usually causes damage to the target cells. Since the total numbers of cells need to reach 10^6 (SSCs $\geq$1 %), the extensive use of FACS may be limited due to the scarcity of SSCs.

MACS was established as a solution to FACS, in order to overcome some of the limitations of the latter. In this method, magnetic microbeads are conjugated directly or indirectly to the specific antibodies, which can recognize a particular antigen expressed on the SSC surface; purification of SSCs is achieved by separating the magnetic microbeads in a high-intensity, gradient magnetic field. A number of markers can be used for MACS-based SSC enrichment, such as EPCAM, β1- and α6-integrin, CD9, GFRα-1 and THY-1 (Feng et al. 2002; Gautam et al. 2012; Kanatsu-Shinohara et al. 2012b), and the separation efficiency is determined by the antigen–antibody affinity. SSCs were enriched by MACS using the anti-Thy-1 antibody from pups. The enriched SSCs were transplanted into infertile recipient mice and progeny were produced 110 days after transplantation (Kubota et al. 2004b). Additionally, flow cytometric analysis revealed that 28 % of the selected cells expressed CD9 after SSC purification by MACS with an anti-CD9 antibody, which indicates a six or sevenfold enrichment compared with the unselected testis cells (Kanatsu-Shinohara et al. 2004).

Since the establishment of MACS, SSC purification has become fast and simple; it no longer relies on large proprietary equipment, and neither is it limited by the initial cell numbers. Even if the SSC number is as low as 1 in 1×10^8 cells, SSCs can be easily sorted by MACS (Gautam et al. 2012; Schmitz et al. 1994). After positive sorting by MACS, the antibody-antigen complex does not dissociate; therefore, the antibody used in a previous purification can interfere with the adhesion of another antibody that combines later with the antigen. Therefore, multi-parameter positive selection cannot be performed with MACS. This is one disadvantage of single-positive MACS selection compared to multi-parameter FACS. Thus, overcoming this limitation of MACS should be a focal point of SSC purification research in the future.

SSC culture

Replacement of the serum culture system with a serum-free culture system

In vitro culture of mouse SSCs was first established at the beginning of the 1990s, and has since then undergone several changes, from high concentration of serum to low concentration of serum, and currently, serum-free culture is being explored (Table 1) (Kanatsu-Shinohara et al. 2003, 2005a; Nagano et al. 1998). Serum contains plasma proteins, polypeptides, growth

Table 1 The research progress in SSCs culture system of mouse

BSA	Serum	Feeder	Result	References
−	+	+	>134 days	Kanatsu-Shinohara et al. (2003)
+	−	+	>2 weeks	Kubota et al. (2004b)
+	−	+	>2 months	Kanatsu-Shinohara et al. (2005a)
+	+	−	>6 months	Kanatsu-Shinohara et al. (2005b)
+	−	−	Produce offspring but germline potential of SSCs reduce	Kanatsu-Shinohara et al. (2011)
−	−	+	Establish a long-term culture system	Aoshima et al. (2013)

factors, hormones, binding proteins, and contact and extension factors that protect cells from damage when they adhere to the culture plates; moreover, it may also contain some unknown factors that may inhibit SSC growth. Under in vivo conditions, these antagonistic factors probably act in synergy to maintain physiological balance; however, this may not be the case in in vitro culture. When mice SSCs were cultured for 2–3 days in a low concentration (0.3–2 %) of serum in vitro, SCs began to develop obviously and most of the SSCs were found attached to the SCs, and SSC colonies began to form till 5–7 days. However, when a high concentration (5–15 %) of serum was added, the mice SSCs still proliferated on SCs and formed colonies, but they eventually disappeared owing to the extensive growth of SCs (Kanatsu-Shinohara et al. 2005a). Thus, high serum concentration may promote the formation of SSC colonies and simultaneously stimulate the extensive growth of SCs, which subsequently inhibits the propagation of SSCs. A study on in vitro goat SSC culture showed that SSCs could proliferate and were maintained for 1 week at serum concentrations as low as 1 %, while all the other high serum concentrations had detrimental effects on SSC expansion (Bahadorani et al. 2012); moreover, some unknown components in serum could induce the differentiation of SSCs (Barnes and Sato 1980). The contradictory functions of serum are probably attributable to the unknown components, difference in contents between different serum batches, and some unknown mechanisms. To overcome these disadvantages, several substitutes for serum are being explored. Serum-free but supplemented with 0.2 % Bovine Serum Albumin (BSA) culture of SSCs of DBA/2J, C57BL/6 strains has been reported (Kubota et al. 2004b), and recently, one study demonstrated that Knockout Serum replacement (KSR) supported the continuous growth of SSCs in vitro without BSA and serum (Aoshima et al. 2013). So, serum-free culture of SSCs has become the main focus of SSC culture research.

Feeder-free SSC culture

Different kinds of feeders have different effects on SSC culture. SCs, SIM mouse embryo-derived thioguanine and ouabain resistant (STO), mouse embryonic fibroblasts (MEFs), mouse testicular stromal cells (MTS), etc., are usually used as feeders after mitomycin (MC) or radiation treatment. SSCs and SCs can coexist under physiological conditions in vivo; however, SSC maintenance in mice is significantly lower when SSCs are cocultured with some SC lines (e.g., SF7 and TM4) compared to other feeder cell types (Nagano et al. 2003). Another study showed that SSCs from rats can be co-cultured longer with SCs than with STO feeders (Hamra et al. 2004). These contrasting results need to be further explored. STO feeders are used more extensively than other feeder cells. For example, when cultured on STO feeders, SSCs from DBA/2J mouse could continuously proliferate for more than 6 months without loss of function (Kubota et al. 2004b). Bovine SSCs were cultured for 7 days, and then colonies were harvested and cultured on four different feeders, STO, MEFs, bovine Sertoli cells (BSCs) and laminin (on a laminin-coated plate) for a short time; STO feeders were found to be a suitable feeder layer for in vitro propagation of bovine SSCs (Nasiri et al. 2012). Moreover, co-culture of STO feeders with hematopoietic stem cells and embryonic stem cells also showed good results (Nagano et al. 1998). A previous study showed that MTS could be used instead of MEFs to generate SSC colonies in C57BL/6 mice (Seandel et al. 2007); however, no significant difference between MEFs and MTS was found for cultivation of SSCs in another

study (Guan et al. 2009). Thus, self-renewal and proliferation of SSCs vary with different kinds of feeders, and the different sources of SSCs and feeders may therefore be important factors that affect SSC growth in culture. Therefore, it is vitally important to choose suitable feeders to culture SSCs from different sources. One of the limitations of using feeders is that the function of feeders varies among cell batches, different inactivated drugs and their dosages, and radiation time. Moreover, some factors secreted by feeders may negatively influence the proliferation of SSCs. All these factors make feeders less stable and more difficult to control.

The use of the laminin-coated plate system may solve the problems encountered with feeders. The plates are convenient to prepare; the components are clear; and this cultivation system is stable. SSCs cultured on feeders generally form clumps, but when transferred to laminin-coated plates they tend to form various types of colonies, ranging from chains to clumps. Chain-type colonies resemble the proliferative patterns of SSCs observed in vivo after transplantation (Kanatsu-Shinohara et al. 2005a; Nagano et al. 1999). Moreover, the morphological changes in SSCs indicate that the cells are closer to the physiological state when cultured on laminin. The SSCs cultured on laminin are significantly different from the SSCs cultured on MEFs. The quality and quantity of SSCs surface markers were altered: not only was the expression of c-kit reduced, but also, the expression of SSEA-1 (not expressed on spermatogonia) was induced (Cooke et al. 1993; Kanatsu-Shinohara et al. 2005a). Thus, the feeder-free culture promotes research on the self-renewal and differentiation of SSCs and opens up new avenues for SSC cultivation in mice (Table 1); moreover, this culture system could possibly be used in farm animals too.

Function of cytokines in SSC self-renewal and proliferation

SSC self-renewal is driven by several cytokines that act in coordination, which make their functions complex and make it more difficult to understand the mechanisms underlying this process (Lee et al. 2009). One study showed that as a spermatogonia-specific transcription factor in the testis, plzf is required to regulate self-renewal and maintenance of the stem cell pool (Costoya et al. 2004), other data had suggested that GDNF could promote the proliferation of SSCs (He et al. 2008). In the next year, a study has suggested that higher concentrations of GDNF (20 ng/ml) should be used for initial culture of SSCs, as it is beneficial for SSC proliferation; however, for long-term maintenance of SSCs, a lower concentration (4 ng/ml) of GDNF can be used (Guan et al. 2009). These results were confirmed by another study, which showed that a combination of 20 ng/ml GDNF and 1,000 U/ml leukemia inhibitory factor (LIF) could significantly enhance the in vitro proliferation of mouse SSCs (Wang et al. 2014). In the presence of GDNF, SSCs from DBA/2J strain mice formed densely packed clumps of cells and continuously proliferated. However, SSCs from other strains of mice required the addition of soluble GFRα-1 and basic fibroblast growth factor (bFGF) for replication; the amount of SSCs in these cultures doubled every 5–6 days, and the clump-forming cells strongly expressed Oct-4 (Kubota et al. 2004b). Similarly, Kanatsu-Shinohara used the basal culture medium StemPro-34 SFM, which is supplemented with epidermal growth factor (EGF), LIF, bFGF, GDNF, 1 % fetal calf serum and so on, to establish a long-term SSC culture system. However, colony formation was observed in ICR and C57BL/6 × DBA/2 F1(BDF1) mice but not in mice with a C57BL/6 or 129/Sv background (Kanatsu-Shinohara et al. 2003). These results indicate that the genetic background of SSCs influences the effect of cytokines on SSC self-renewal and therefore plays a role in the performance of SSC cultures. Therefore, future research must focus more on the inherent self-renewal mechanism of SSCs.

Challenges faced in serum- and feeder-free culture systems for mouse SSC

The SSC culture system is affected by the serum and feeder cells used, so investigations on serum- and feeder-free culture systems are underway. A serum-free culture system for SSCs from mice was developed successfully by using STO feeders and α-MEM culture medium supplemented with bFGF and GDNF. The study showed that proliferation of SSCs at day 14 was decreased significantly at all concentrations of FBS compared to this serum-free medium (Kubota et al. 2004b). In 2005, Kanatsu-Shinohara and his colleagues found that SSCs could expand in serum-free conditions on MEFs; moreover, they found that

SSCs could be successfully cultivated without feeder cells on a laminin-coated plate. However, SSCs could not expand when both serum and feeder cells were absent (Kanatsu-Shinohara et al. 2005a). In 2011, a serum- and feeder-free culture system was finally developed in their lab for long-term propagation of SSCs. Although, the SSCs could be used to produce offspring after germ cell transplantation, the germline potential of the SSCs was reduced (Kanatsu-Shinohara et al. 2011). Bovine type A spermatogonial cells were cultured with different concentrations of fetal calf serum (FCS): the results showed that 80 % of the cells were alive and proliferating in the presence of 2.5 % FCS after 1 week of cultivation, while only 20 % of the cells were alive in the absence of serum; moreover, higher concentrations of FCS only enhanced the number of somatic cells (Izadyar et al. 2003). Therefore, there is a need to further explore different serum- and feeder-free cultures and to establish effective culture systems. Future studies should focus on how to maintain the high activity and reproductive potential of SSCs with serum- and feeder-free culture systems.

Identification of SSC

Identification of SSC using the transplantation method

Difficulty in the identification of SSCs owing to a lack of special biological markers for SSCs has posed a bottleneck in the study on SSCs. Testis cell transplantation, a powerful method for functional identification of SSCs in vitro, was first used in Brinster's study (Brinster and Zimmermann 1994). In this method, mutant recipients and normal mice treated with busulfan are used as recipients (Johnston et al. 2000); then, donor cells carrying reporter genes (e.g. EGFP, green fluorescent protein [GFP]) are transplanted into recipient testes to examine whether cultured SSCs could restore fertility by observing donor-derived offspring or detecting donor-derived genes (Guan et al. 2009; Kubota et al. 2004b). In Ma's study, donor cells expressing EGFP were transplanted into heat shock-treated recipients, and healthy EGFP-expressing offsprings were obtained by intracytoplasmic injection of round spermatids recovered from heat shock-treated recipients after their fertility was restored (Ma et al. 2011); this method could also be used to study the reliability of the reconstruction of spermatogenesis in infertile recipients. However, the transplantation method requires more manpower, is more expensive, and also requires more time. Therefore, it cannot be widely used for SSC identification, and it is necessary to explore other more efficient methods for SSC identification.

Identification of SSC using immunohistochemical staining

To identify SSCs, immunohistochemical staining for protein markers of stem cells is another technique in use. In the study by Ryu et al., antibodies against OCT4, RET receptor kinase, and neural cell adhesion molecule (NCAM) were used as the primary antibodies, and fluorescein isothiocyanate (FITC)-conjugated proteins were used as the secondary antibodies were used as the secondary antibodies. Green fluorescence was detected in SSC clumps but not detected in STO feeder cells. Moreover, OCT4 was found to be located at the cell nucleus by laser scanning confocal microscopy (Ryu et al. 2005). Also, Inhibitor of DNA binding 4 (ID4), a recently identified SSCs marker in the mouse, could be used as an antigen (Oatley et al. 2011a). Although useful, one of the limitations of this method is the observation of nonspecific staining. Moreover, fluorescence intensity can be influenced by staining time and method, and the antibody concentration needs to be further optimized.

Identification of SSC using reverse transcription-polymerase chain reaction

In this method, SSC samples are randomly selected and total RNA is isolated. cDNA is established with Oligo (dT) or random primers with the help of reverse transcriptase, and then amplified by PCR. The quantity and quality of SSCs can be determined from the amplification results. The results for SSC samples co-cultivated with SCs using reverse transcription-polymerase chain reaction (RT-PCR) showed that *GATA4* and *PLZF* were highly expressed while c-Kit was lowly expressed or not expressed at all in SSCs (Kanatsu-Shinohara et al. 2005b, 2012a; Oatley et al. 2011b). To identify SSCs at the transcriptome level using RT-PCR, *OCT4* and *PLZF* are used as stem cells markers, *c-Kit* is used as a marker for differentiating spermatogonia, either *GAPDH* or *β-actin* is used as a

reference gene, and *GATA4* is used as a special control for SCs. Although RT-PCR is a simple method that is widely used in SSC identification, it can be costly and time consuming, as well as tedious to perform.

Identification of SSC using flow cytometric analysis

The principle for this technique is similar to that for immunohistochemical staining; the antibody is marked with fluorophores before SSCs are identified using flow cytometric analysis. After the marked antibody is incubated with the SSCs, the samples are analyzed using flow cytometry. During analysis, the fluorescence signals from SSCs are transformed into electrical signals that are analyzed. In this method, specific antibodies against SSC markers such as β1- and α6-integrin, CD9, and EpCAM are used, after conjugation of the antibodies with dyes such as allophycocyanin, phycocerythrin, and FITC (Kanatsu-Shinohara et al. 2005b; Shinohara et al. 1999). Besides identification, purification of SSCs is also possible using flow cytometric analysis, and therefore the proliferation of SSCs can also be assessed. Unlike other methods, culture of the SSC samples can be continued and the SSCs can be transplanted into infertile recipients after analysis. Therefore, flow cytometric analysis seems likely a viable option for SSC identification, providing specific markers of SSCs are developed.

Identification of SSC using the transgenic animals and the related technique

Since there are limited numbers of SSC-specific surface markers, we also introduce alternative technique for studies on spermatogenesis. Recently, developments of transgenic animals were reported such as GFP-pig (Kawarasaki et al. 2009), transgenic marmoset (Sasaki et al. 2009), and diabetes-model pig (Umeyama et al. 2009). In spermatogenesis studies in transgenic mouse, there are reports about combination use of Sl/Sld knockout mouse with c-kit ligand, KITL (also called as stem cell factor: SCF), Acr-GFP mouse and Gsg-GFP mouse (Sato et al. 2011a, b, 2012). In Acr-GFP mouse and Gsg-GFP mouse, GFP fluorescence is expressed only in the appropriate phase of meiosis and the fluorescence can be observed with stereomicroscopes. The KITL Sl/Sld mouse is known to have a defect to make mature sperm and they only have a small number of spermatogonia. Immature SSCs can be differentiated using an ex vivo tissue culture technique. Immature SSCs are co-cultured with KITL and colony stimulating factor in this ex vivo culture. KITL Sl/Sld mouse-derived immature SSCs are then differentiated into mature sperms. The study on spermatogenesis using transgenic animals might help to identify the cultured SSCs. Although there is some hysteresis, we may also obtain some verifications through tracing the development state and GFP expression state of SSCs which were cultured during their spermatogenesis process.

Future prospects

In recent years, great progress has been made in culture techniques for SSCs, especially mouse SSCs; these methods provide a theoretical basis for exploring SSC culture in farm animals. However, there are some drawbacks to these methods, the main one being the lack of specific markers for SSCs. Another limitation is the low efficiency and replicability of SSC enrichment methods. Therefore, future studies in this field should focus on discovering SSC-specific markers and optimizing the method for enrichment and identification of SSCs. Moreover, studies on the biological characteristics of SSCs and their self-renewal mechanism can contribute to overcoming the bottleneck in the enrichment, culture and identification of SSCs.

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