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Spermatogonial Stem Cells and Animal Transgenesis

Flavia Regina Oliveira de Barros,
Mariana Ianello Giassetti and José Antônio Visintin
School of Veterinary Medicine and Animal Sciences – University of Sao Paulo
Brazil

1. Introduction

Spermatogonial stem cells (SSCs) are unipotent adult stem cells responsible for the maintenance of the spermatogenesis throughout the entire life of the male. We could say that the mammalian spermatogenesis is a classic adult stem cell-dependent process, sustained by self renewal and differentiation of SSCs. They are the only germline stem cells in adults. These cells can be found in the seminiferous tubule, lying near to the basement membrane. The SSC may choose to self-renewal or generate a daughter cell committed to differentiation. Studying SSCs provides a model to better understand adult stem cell biology and decipher the mechanisms that control SSC functions. It was reported that these cells hold the ability to colonize the seminiferous tubules after transplantation, restoring spermatogenesis. Besides the biomedical potential to perform studies of infertility in many species, SSCs present a promising application in biotechnology in the production of transgenic animals. This alternative route for transgenesis is of interest because a single male will generate by regular mate a variety of transgenic progenies. The production of a transgenic gonad can overcome the obstacles faced with the sperm-mediated gene transfer (SMGT) due to the high specialization of sperm. The use of SSC for transgenesis relies on targeting a much more undifferentiated germ cell and the potential permanent modification of the germ line. In this manner, this chapter aims to review the following topics regarding SSCs: (1) Mammalian spermatogenesis and SSCs; (2) Characterization of SSCs; (3) Isolation and *in vitro* culture of SSCs; (4) Transplantation of SSCs and animal transgenesis.

2. Spermatogonial stem cells and spermatogenesis

Spermatogenesis is a highly organized and complex process that is responsible for sperm production in male individuals (Russell et al., 1990). Besides providing continuous source of spermatozoa, it is responsible for maintenance of its stem cell population by constant replication of SSCs. In mammals, millions of sperm cells are produced everyday from SSC (Meistrich & van Beek, 1993). In the testis, only SSCs hold the self-renewal ability, i.e. the ability to undergo a series of mitotic cycles without differentiating. In this manner, we can say that spermatogonial stem cells are at the foundation of spermatogenesis. They are the adult stem cell population of the testis, which is responsible for the maintenance of spermatogenesis throughout the entire life of the male. As observed in other tissue-specific

stem cells, SSCs are rare, being only 0.03 percent present in an adult mouse testis (Tagelenbosch & de Rooij, 1993). They are present in the testis in such a small number due to the high density of differentiated germ cells, as differentiating spermatogonia, spermatocytes, spermatids and sperm, all originated from SSCs. We define SSCs as stem cells based on their ability to balance self-renewal and differentiation. The self-renewal sustains the stem cell pool at the testis because SSC undergo multiple mitosis producing new SSCs. These new SSCs hold the same self-renewal and differentiation potential as their precursors. Upon demand, SSCs start the cell division in order to produce a differentiated daughter cell. The balance of these two cell divisions maintains spermatogenesis, which can produce millions of sperm each day without causing depletion of cell source.

SSCs originate from primordial germ cells (PGC), which migrate from the embryonic ectoderm in the epiblast, through the allantois and hindgut until reaching genital ridges (Lawson & Pedersen, 1992; Clark & Eddy, 1975). Once PGCs colonize the genital ridge, they are enclosed by differentiating Sertoli cells, starting the formation of seminiferous cords, that will eventually give rise to seminiferous tubules (Byskov & Hø´yer, 1994). From this stage on, the germ cells are called gonocytes because they differ morphologic from PGCs (Clermont & Perey 1957; Huckins & Clermont 1968). In the late stage of gestation, the gonocytes undergo proliferation and become quiescent, i.e., arrested in the G0/G1 phase of the cell cycle (Clermont & Perrey, 1957). These cells remain in mitotic arrest until the peri-pubertal period, when they start proliferating again, this time to produce type A SSCs. Increasing levels of gonadotrophic hormones concentration triggers this massive proliferation of type A SSCs, marking the onset of spermatogenesis (Huckins & Clermont, 1968; Belveé et al., 1977).

It is known that in rhesus monkey (de Rooij et al., 2002) and human (Clermont, 1966), two subtypes of type A spermatogonia are morphologic distinguishable: A_{dark} and A_{pale}. The A_{dark} spermatogonium act as a true SSC, forming the testis regenerative reserve while A_{pale} has a progenitor role, constituting the functional reserve (Ehmcke et al., 2006). In rhesus monkey, these spermatogonia are followed by four generations of spermatogonia in different stages of differentiation (B_1,B_2, B_3 and B_4; de Rooij, 1986). In human, only one generation of type B spermatogonium can be observed before formation of spermatocytes (Clermont, 1966). In rodents, seven subtypes of type A spermatogonia have been reported: A_{simple} (A_s), A_{pared} (A_{pr}), $A_{aligned}$ (A_{al}), A_1, A_2, A_3 and A_4 (Huckins, 1971a,b; Huckins & Oakberg, 1978). A_s spermatogonia are considered the SSCs. Although still undifferentiated, A_{pr} and A_{al} produce expanded colonies of SSCs because they have already undergone mitosis. However, due to a formation of an intercellular bridge that connects their daughter cells, their division is considered incomplete (Zamboni & Merchant 1973). As a result, these cells no longer possess the self-renewal ability as subtype A_s spermatogonia. Subtypes A_1-A_4 spermatogonia also constitute expanded SSCs colonies, but differently from A_{al} and A_{pr}, they are already synchronized with the cycle of the seminiferous epithelium. In this manner, it is possible to say that A_1-A_4 spermatogonia are already committed to differentiation into future spermatozoa. We refer as the cycle of seminiferous epithelium the synchronic evolution of germ cells from one stage of spermatogenesis to the next. In other words, the cycle of seminiferous epithelium is the completion of ordered events of cell association, divisions and stages in the seminiferous epithelium over time (Russell et al., 1990). In this cycle, the succession of spermatogonia, spermatocytes and spermatids from basement

membrane toward the lumen of seminiferous tubule is established in a stepwise manner during postnatal development.

In bovine, a livestock species, a similar classification was proposed in 1995 by Wrobel et al. According to this classification, there are basal stem cells, corresponding to type A_s and A_{pr} spermatogonia in rodents, aggregated spermatogonial precursor cells, equivalent to A_{al} spermatogonia and finally committed spermatogonial precursor cells, equivalent to A_1-A_4 spermatogonia. It was suggested that type A_{pr} spermatogonia also hold stem cell properties in bulls.

3. Characterization of spermatogonial stem cells

SSC are the foundation of the productive spermatogenesis that results in the continuous production of spermatozoa in the postnatal life, but studies with SSCs are complicated because these cells are few in number and no unique identifying characteristics have been reported to date. Thus, little is known of their morphology, functional assay or biochemical characteristics and those evaluations become harder in postnatal tissue. Togelenbosch and de Rooij (1993) performed a quantitative study with spermatogonial cells in mouse testis comprising 1 in 3333 cells from adult mouse testis.

In the spermatogenic cycle with each division the number of cells theoretically double, but is important to remember that generally there is no divisions between A_{al} to A_1 cells. Although morphological changes occur and A_1 cells slightly resemble A_{al} cells. Only some spermatogonial cell types can be distinguished by morphologic characteristics and this may actually cause many disturbances in spermatogonial kinetics studies. In almost all species the type A have very similar morphologic characteristics when these cells are analyzed in whole seminiferous tubules. On the other hand, the type A (as a class), Intermediate and type B spermatogonia can be distinguished by minor morphological changes, using either light or electron microscopy (Russell et al., 1990).

The type A spermatogonia have two different surfaces: one flattened and another rounded. The first surface acquires this format because of its direct contact with basal lamina and the second surface is surrounded by Sertoli cells. In the nucleus is observed little presence of heterochromatin and the nucleolus is visible. The Intermediate spermatogonia typically show an ovoid nucleus, present more heterochromatin located close to the nuclear envelop compared to type A and have also a rounded and a flattened surface. Finally, type B spermatogonia has a rounded nucleus with a moderated quantity of heterochromatin allocated around nuclear edge. A smaller part of the cellular membrane is in contact with basal lamina then the most part of surface is rounded. Thus, main morphological aspects that are analyzed to distinguish the types of spermatogonial cells are, first, the amount of heterochromatin in the nucleus and its relation to nuclear membrane. The type A basically has no heterocromatin, Intermediate displays a moderate quantity and type B an abundant amount. The second important aspect is that spermatogonial cells are part of seminiferous epithelium and always have a flattened surface in contact with basal lamina, and rounded, in contact with Sertoli Cells (Russell et al., 1990). Approximately 300,000 cell (types A_s, A_{pr}, A_{al} and Intermediated) were counted and characterized from mice seminiferous tubules (Togelenbosch & de Rooij, 1993) being identified approximately 35,000 type A_s cells from each testis (Meistrich & van Beek, 1993). The morphological evaluation is an important tool

for permatogonial studies but it provides many disturbance in the analysis of data, mainly in cells that is analyzed outside of seminiferous tubules environment. Nevertheless, espermatogonial cells can be identified with functional assays or molecular techniques besides the morphological characterization.

In the functional assay, the presence of SSC, for example: from a new purification protocol, was checked by the transfer of progenitor germ cell to the testis of a recipient animal. Spermatogenesis of the recipient testis was previously depleted by the treatment with an alkylating agent, Busulfan or fractionated X-irradiation (local testicular doses of 1.5 and 12 Gy, 24 h apart; Aponte et al., 2005). After transplantation, SSC repopulate the recipient animal seminiferous tubules, that produces a spermatic cycle with donor progenitor cells from the same specie or not.

Whilst in the undifferentiated stage, SSCs express different proteins and genes. In both cases, they are not produced in greater differentiation (Type A_1-A_4, Intermediated, spermatocytes and spermatids; Caires et al., 2010). A key step in studying the biology of SSCs is to determine their gene expression profile. However, a scarce knowledge of molecular markers has been accumulated in recent years (Kokkinaki et al., 2010). Some research groups have demonstrated that glial cell-.derived neurotrophic factor (GDNF) is the most essential factor for SSC self-renewal and *in vitro* maintenance in rodents (Caires et al., 2010; Ryu et al., 2005; Meng et al., 2000; Kubota et al., 2004a,b; Kanatsu-Shinohara et al., 2005; Kanatsu-Shinohara et al., 2008; and Braydish-Stalle et al., 2005) and that GDNF receptor (GFRA1) is expressed by SSC/progenitor cells (Naughton et al., 2006; Hofmann et al., 2005 and He et al., 2007). The activation of GDNF pathway probably is related with other pathways that promote the proliferation and the self-renewal of SSCs (Caires et al., 2010; Jijiwa et al., 2004; Braydich-Stolle et al., 2007; Oatley et al., 2007 and Lee et al., 2006). Thereby, many molecular markers for SSC are associated with GDNF pathway.

4. SSC markers and differences among species

The establishment of molecular signatures for SSCs are a complex and difficult process but some molecular markers have been defined for SSCs and undifferentiated spermatogonia (Caires et al., 2010). It is important to know that all these markers (Table 1) were established for different species using a pool containing undifferentiated germ cells.

One of the most important molecular marker for progenitor germ cells is the GRFA1 that is a co-receptor of RET for the GDNF (He et al., 2007). GDNF is related to neural development (He et al., 2007 and Garces et al., 2000) and renal morphogenesis (He et al., 2007 and Vega et al., 1996). In spermatogonial cells, this factor plays an important role in the regulation of proliferation and differentiation or undifferentiated (He et al., 2007, Naughton et al., 2006; Takakoro et al., 2002; Meng et al., 2000 and Hofmann et al., 2005). Others important markers are Nanog and Pou5f1 (Oct3/4). They are essential transcription factors for the maintenance of pluripotency (Goel et al., 2008). THY-1, a member of the Ig super family is highly expressed in rat stem cells and in SSCs from pre-pubertal bulls (Aponte et al., 2005, Ryu et al., 2004 and Reding et al., 2010), However, the role of THY-1 in the male fertility is still unknown (Aponte et al., 2005 and Barlow et al., 2002). PLZF (*Zfp145*) is a molecular marker for A_s, A_{pr} and A_{al} spermatogonia and as GRFA1, it is related with self-renewal of SSC (Aponte et al., 2005; Buaas et al., 2004 and Costoya et al., 2004). NGN3 also is expressed in

the same cell types as PLZF and acts in the differentiation of spermatogonia (Aponte et al., 2005 and Yoshida et al., 2004), but also is present in spermatocytes (Aponte et al., 2005 and Reverot et al., 2005).

Molecular Marker	Author	Animal
Bcl6b	Oatley et al., 2006	mouse
CD49f (alpha 6 integrin)	Izadyar et al., 2011 Maki et al., 2009 Alipoor et al., 2009	human primate mouse
DBA	Izadyar et al., 2002	bovine
Etv5 (erm)	Oatley et al., 2007 and Schlesser et al., 2008	mouse
Gfra1 (Gfra1)	Naughton et al., 2006	mouse
GPR 125	Izadyar et al., 2011 Seandel et al., 2007	human mouse
Lhx1	Oatley et al., 2007	mouse
NANOG	Goel et al., 2008 Fujihara et al., 2011 Sada et al., 2009	swine bovine mouse
Neurog3 (Ngn3)	Yoshida et al., 2004	mouse
PGP 9.5	Goel et al., 2010	mouse
Pou5f1 (oct4)	Pesce et al., 1998 Fujihara et al., 2011	mouse bovine
Ret	Naughton et al., 2006	mouse
SSEA4	Izadyar et al., 2011 Maki et al., 2009	human primate
THY1	Maki et al., 2009; Reding et al., 2010 and Herrid et al., 2007 Fujihara et al., 2011	primate bovine
UCHL1 (PGP9.5)	Fujihara et al., 2011	bovine
Utp14b	Boettger-Tong et al., 2000 and Shetty et al. 2006	mouse
VASA	Fujihara et al., 2011	bovine
Zbtb16 (Plzf)	Buaas et al., 2004 Reding et al., 2010	mouse bovine

Table 1. Spermatogonial cells molecular markers in different species

5. Isolation and *in vitro* culture of spermatogonial stem cells

5.1 Isolation techniques

As discussed earlier, SSCs are found close to the basement membrane and their presence in the adult testis is restricted to less than 0.1 percent of all germ cells (Togelenbosch & De Rooij, 1993). In this manner, the election of the most suitable technique to isolate them is very important to establish an *in vitro* culture of SSC. Nowadays, the two step enzymatic digestion is the most popular technique used to isolate SSCs. This technique is based on two incubations of testicular tissue fragments in the presence of enzymes to digest it. It was first proposed by Davis and Schuetz, (1975) in rats and Bellvé et al., (1977) in mice.

The enzymatic digestion have been adapted and applied to many other species since then. The isolation of SSCs is often followed by a purification or enrichment step, in order to increase the amount of SSCs in the cell culture. For that, many approaches have been reported, including the discontinuous Percoll density gradient (Van Pelt et al., 1996), differential plating (Izadyar et al., 2002), flow cytometry cell sorting and magnetic cell sorting using SSCs specific antibodies. We can highlight the Percoll gradient as the most popular enrichment protocol for SSCs. However, many modifications have been proposed to this technique regarding the Percoll density adopted and number of layers used to prepare the gradient. The differential plating consists on the overnight *in vitro* culture of freshly isolated SSCs followed by subculture of only non adherent cells. Germ cells tend to remain in suspension, while supporting cells and other testicular cells adhere to the culture dish. These two techniques are usually combined in order to enrich the population of SSCs.

Other important factor in SSCs isolation if the age of donor individuals at the moment of germ cell isolation. Kanatsu-Shinohara et al. (2004) isolated SSCs from newborn mice (0-2 days of age) because at this age, the most primitive types of spermatogonia are predominant in the testis. When Guan et al. (2006) isolated SSCs from mice with 4-6 weeks of age, they obtained a less pure population of SSCs. Similar results were obtained by Seandel at el. (2007) with 3-5 weeks mice. We believe the same happens in livestock species, as bovine. Izadyar et al. (2002) isolated 65-87% type A SSC population from 5-7 months calves.

5.2 *In vitro* culture of SSCs

In vitro culture of SSCs faces similar hurdles to those commonly observed in *in vitro* culture of adult stem cells. However, many advances have been achieved in this area, and we can find in the literature protocols with satisfactory outcomes.

It was first reported that SSCs can be *in vitro* cultured for months by Nagano et al. (1998). The same group later suggested the addition of GDNF is important to short-term *in vitro* maintenance of SSCs (Nagano et al., 2003). Aponte et al. (2006) demonstrated the importance of this growth factor in the *in vitro* culture of bovine SSCs. Kanatsu-Shinohara et al. (2003a,b) studied the dynamics of gonocytes throughout *in vitro* culture, assessing the cell number increasing. In this study, it was possible to observe a 10^{14}-fold increase in cell number. In 2005, Kanatsu-Shinohara et al. developed a serum-free culture condition, when germ stem cells were cultured *in vitro* over 6 months. Serum-free conditions to culture SSCs have been optimized in rodents (Kubota et al., 2004a,b and Ryu et al., 2005) in order to support long-term maintenance.

The co-culture of SSCs with monolayers of other cell types as mitotically inactivated murine embryonic fibroblasts (MEF) or STO feeder cells (Nagano et al., 1998) are still discussible, presenting different results so far. MEF is widely used in *in vitro* culture of embryonic stem cells (Evans & Kaufman, 1981) and has been applied as feeder cells to murine SSCs cultures (Kanatsu-Shinohara et al., 2004a). Oatley et al., (2002) cultured bovine SSCs over a monolayer of STO feeder cells and in 2004, the same group developed a lineage of bovine embryonic cells (BEF), which was shown to be effective in the *in vitro* maintenance of bovine SSCs. However, Lee et al. (2001) and Aponte et al. (2006) successfully cultured bovine SSCs

under feeder-free conditions. Izadyar et al. (2003a) adopted a lamimin based extracelular matrix to support bovine SSCs *in vitro*.

5.3 Cryopreservation of SSCs

There are two ways to preserve SSCs, long-term *in vitro* culture and cryopreservation. Culture and cryopreservation in combination could be used to immortalize a male's genetic line through the germ cells due to the spermatogonial stem cells ability to self-replicate (Oatley et al., 2004). Since it is still hard to maintain pure populations of SSCs for long periods under in vitro condition, the development of effective cryopreservation protocols have been considered of high interest. Izadyar et al., (2002) cryopreserved type A bovine SSCs using DMSO obtaining a survival rate of 50% after thawing cells. Kaul et al., (2010) also adopted DMSO as SSC cryoprotectant in caprine, also observing a 50% survival rate after cryopreservation. Due to the fact enrichment protocols for type A SSCs are still being improved, many groups adopted the cryopreservation of testicular tissue instead of isolated cells. It appears to provide good results in preserving germ cells (Sato et al., 2011; Kaul et al., 2010).

6. SSCs transplantation and transgenesis

The development of male germ cells transplantation methods provided a powerful means to study the biology of SSCs and its role in spermatogenesis and opened a door to a new potential tool for transgenesis. In addition, the testis cells transplantation is considered the unique *in vivo* functional assay for SSCs. This technique was first used to verify the function of *in vitro* cultured SSCs in mice (Brinster & Zimmermann, 1994; Brinster & Avarbock, 1994). Testicular cells are isolated from a fertile donor and microinjected into the seminuferous tubule of an infertile recipient. It is expected to observe the resumption of spermatogenesis from a donor SSC-derived colonies in the recipient testes. These cell colonies rise from a single transplanted SSC, what allows the quantification of these clonal events (Brinster, 2002). Besides the possibility to study male infertility, the transplantation of SSCs also provides another way to conserve reproductive potential of genetically valuable individuals within or between species and, finally, can be used to produce transgenic animals after generation of transgenic sperm cells. This last application is especially important for species in which embryonic stem cell lines have not been established and other transgenic techniques present limited efficiency. Transgenic animals have huge applications from basic science such as the creation of animal models for human diseases, like Parkinson´s (Crabtree & Zhang, 2011) to production of recombinant pharmaceutic proteins in the animal's fluid: blood, milk (Houdebine, 2000a,b and Houdebine, 2002), egg white (Zhu et al., 2005; van de Lavoir et al., 2006 and Lillico et al., 2007) and seminal plasma (Dyck et al., 2003). Ever since the generation of the first transgenic animal, in 1980, through pronuclei microinjection in an embryo's pronuclei (Houdebine, 2009), this method has been used in other prolific species as rat, rabbit and pig (Houdebine, 2000a,b). However, along the years, many disadvantages of pronuclei microinjection were reported. One of the most important is the misplaced injection of DNA in the cell cytoplasm and not in its pronucleus. Additionally, the exogenous DNA interaction with the host cells genome is quite variable (Houdebine, 2009). Alternatively, other techniques were developed, such as: gene transfer with transposons, lentiviral vectors, sperm, pluripotent, stem and somatic cells. In 2002, Lavitrano et al. obtained a large number of transgenic pigs using sperm-mediated gene transfer (SMGT).

The authors reported that up to 80% of the animal had the exogenous gene integrated in the genome, thus SMGT was more efficient than other techniques previously described. SSCs of all mammalian species examined, including human, can replicate in mouse seminiferous tubules following transplantation, the growth factors required for SSCs self-renewal are probably conserved among mammalian species (Kubota et al., 2006).

Although most studies have been performed in rodents, germ cell transplantation has also been applied to non-rodent species as pigs, goats, cattle, monkeys and recently fish and chickens (Honaramooz et al., 2002a,b, 2003; Schlatt et al., 2002; Izadyar et al., 2003b; Takeuchi et al., 2003; Yoshizaki et al., 2005; Lee et al., 2006; Mikkola et al., 2006; Okutsu et al. 2006; Trefil et al. 2006), as reviewed by Dobrinski, (2008). The first hurdle found when germ cell transplantation was applied to livestock species was the differences in testicular anatomy and physiology. While in rodents it is possible to microinject germ cells directly into the seminiferous tubule via efferent ducts, the same is not feasible in larger animals. Thus, the alternative use of ultrasoung to guide the needle during the injection of cells was successfully reported by Kaul et al. (2010) in goats. In bovine, this ultrasound guided needle technique was successfully applied when an autologous transplantation (Izadyar et al., 2002) as well as a heterologous transplantation (Herrid et al., 2006) was performed. It has been demonstrated that germ cell transplantation can be performed more efficiently after suppression of spermatogenesis in recipient animals. The most popular chemical treatment consists of administering a DNA alkylating agent, Busulfan, that destroys proliferating cells. Busulfan is commonly used to suppress spermatogenesis in rodents. An alternative to Busulfan, irradiation of the testis (Creemers et al., 2002; Schlatt et al., 2002), being frequently adopted in studies with large animals as bulls (Izadyar et al., 2003). Despite all the described potential of SSCs to produce transgenic animals, until now, few groups have genetically modified these cells, being the efforts more restricted to laboratory species.

7. Conclusions and perspectives

As discussed in this chapter, SSCs, like every other adult stem cell in mammals, retains the ability of either self-renewal or differentiation. In this case, the differentiation process is known as spermatogenesis. Interest in spermatogonia has grown in recent years as a result of exciting developments in stem cell research in general and the development of new research tools allowing the isolation, culture and transplantation of these cells.

Because there are a low concentration of SSCs in mammal testis and isolation processes are difficult (Meachem et al., 2001), the assessment of biological activity and cell viability are essentials for the maintenance of the SSC (Potten & Loeffler, 1990; van der Kooy & Weiss, 200; Watt & Hogan, 2000). Brinster & Avarbock (1994) performed the first SSC transplantation and reported this technique to be a good functional assay. In this way, the progenitor germ cells would be in the correct environment having direct contact with somatic niches (Brinster, 2002). Because SSCs are capable of restoring spermatogenesis after transplantation into testes which spermatogenesis had been suppressed, their transplantation opened the door to many possibilities of usage. In this context, we can include the preservation and reestablishment of the reproductive potential of the animals. For example, and animal with desirable genetic traits which can no longer mate, can

continue to spread its genetics through germ cell transplantation. In humans, cancer patients now have the opportunity to cryopreserve their SSCs during chemotherapy or radiation treatments. However, there is so much to study and to understand regarding the biology of SSCs before their transplantation in human becomes a routine procedure. When SSC culture becomes available for clinical use, efficient protocols for cryopreservation of these cells and testicular tissue will be of great value.

Finally, the most exciting potential usage of SSCs relies on their capability to transfer genetic modifications to the next generation in a fast manner. The potential use of SSCs in animal transgenesis has attracted the attention of many research groups all over the globe. As discussed, it is almost impossible to describe the value of a transgenic animal, since they can be used in basic science as animal models to human diseases. In addition, transgenic animals can serve as bioreactors, producing proteins of high interest in the human pharmaceutical industry.

In conclusion, SSCs has many future research perspectives, such as: infertility treatment, contraceptive strategy, *in vitro* spermatogenesis, the development of markers for identification of spermatogonial subtypes, innovative research using germ cell transplantation, preservation of fertility for cancer patients, generation of transgenic animals and preservation of valuable animals (Meachem et al., 2001).

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