REPRODUCTION
REVIEW

Spermatogonial stem cells from domestic animals: progress and prospects

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Abstract

Spermatogenesis, an elaborate and male-specific process in adult testes by which a number of spermatozoa are produced constantly for male fertility, relies on spermatogonial stem cells (SSCs). As a sub-population of undifferentiated spermatogonia, SSCs are capable of both self-renewal (to maintain sufficient quantities) and differentiation into mature spermatozoa. SSCs are able to convert to pluripotent stem cells during *in vitro* culture, thus they could function as substitutes for human embryonic stem cells without ethical issues. In addition, this process does not require exogenous transcription factors necessary to produce induced-pluripotent stem cells from somatic cells. Moreover, combining genetic engineering with germ cell transplantation would greatly facilitate the generation of transgenic animals. Since germ cell transplantation into infertile recipient testes was first established in 1994, *in vivo* and *in vitro* study and manipulation of SSCs in rodent testes have been progressing at a staggering rate. By contrast, their counterparts in domestic animals, despite the failure to reach a comparable level, still burgeoned and showed striking advances. This review outlines the recent progressions of characterization, isolation, *in vitro* propagation, and transplantation of spermatogonia/SSCs from domestic animals, thereby shedding light on future exploration of these cells with high value, as well as contributing to the development of reproductive technology for large animals.

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Introduction

Spermatogonial stem cells (SSCs) comprise a sub-population of undifferentiated spermatogonia. Spermatogonia originate from primordial germ cells (PGCs) during embryonic development. In mice, PGCs migrate to the genital ridge at around 10.5 days post coitum, and then they differentiate into gonocytes (Franca *et al*. 2000). After several days of replication, gonocytes arrest at the cell cycle of G_0/G_1 stage. It is only after birth that gonocytes resuming mitosis gradually migrate from the center to the periphery of seminiferous tubules, with the eventual consequence that they reach the basement membrane and become spermatogonia (Culty 2009). Gonocytes, preceding the formation of spermatogonia, represent the transient germ cell population with stem cell characteristics in neonatal testes. Like SSCs, gonocytes are able to colonize and produce donor-derived spermatogenesis after transplantation into infertile recipient testes (Jiang & Short 1998, Orwig *et al*. 2002). In pigs and goats, gonocytes transform to spermatogonia at about 2 months after birth (Byskov & Hoyer 1994), while in sheep and cattle, this transition commences at ∼3 and 4 months after birth respectively (Curtis & Amann 1981, Pedrana *et al*. 2008, Borjigin *et al*. 2010, Sarma & Devi 2012).

The survival and development of SSCs are based on a particular microenvironment termed 'niche' (Scadden 2006). SSCs present in niche strike a balance between self-renewal and differentiation to maintain the consistent spermatogenesis. It has generally been accepted that Sertoli cells, peritubular myoid cells as well as Leydig cells constitute the niche (Kokkinaki *et al*. 2009, Oatley *et al*. 2009). Specifically, Sertoli cells, a kind of polorized columnar epithelial cells, offer nutrients for SSCs and differentiating germ cells and mediate intricate signals to support spermatogenesis. Sertoli cells produce glial cell line-derived neurotropic factor (GDNF) and basic fibroblast growth factor (bFGF), both of which are of paramount importance to SSCs self-renewal *in vitro* (Meng *et al*. 2000, Hofmann *et al*. 2005). Peritubular myoid cells and Leydig cells generate colony-stimulating factor 1 (CSF1) which exhibits a synergic effect with GDNF (Oatley *et al*. 2009). Apart from the ability to

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secrete CSF1, Leydig cells can also stimulate the development of gonad and maintain spermatogenesis via the production of testosterone.

SSCs as a canonical category of stem cells are best characterized by their capability of self-renewal as well as their commitments to the germ line. Recent studies suggest that two functional SSC populations are present in testes: the actual stem cells (SSCs undergoing self-renewal) and the potential stem cells (a cell population exhibiting the ability to self-renew on condition that they are in adversity) (Brinster & Zimmermann 1994, Yoshida 2012). When SSCs experience a vast amount of loss under adverse conditions (e.g. exposure to toxicants or chemical agents, physical impairments), the progenitor cells committed to differentiation may act as potential stem cells and resume self-renewal. Consequently, self-renewal of the whole spermatogonial population tends to outweigh their differentiation, eventually resulting in the recovery of the actual SSCs (Martin & Seandel 2013). Nevertheless, this proposition of actual and potential stem cells is still heavily debated and is a subject of further studies (de Rooij & Griswold 2012).

SSCs not only share some identities with other stem cells but also hold their distinct characteristics. Of most uniqueness is that SSCs are the adult stem cells that could transmit genetic information from the paternal generation to the descendants (Brinster 2002, Hamra *et al*. 2004). Therefore, SSCs are of particular utility in animal genetics, breeding, and reproduction, provided that gene transfection and homologous transplantation of SSCs are synthesized to produce transgenic animals with improved productivity and commercial value. In addition, SSCs provide an ideal model to unravel the mechanisms for stem cell self-renewal and differentiation.

Characterization of spermatogonia in domestic animals

It is a standard practise to characterize SSCs before subsequent culture, transplantation, or manipulation of the cells. Increasing knowledge has been acquired regarding the molecular markers expressed in murine SSCs (e.g. *Itga6*, *Itgb1*, *Gfra1*, *Thy1*, *Cd9*, *Cdh1*, *Lin28*, *Gpr125*, *Zbtb16* (*Plzf*), *Pou5f1* (*Oct4*), *Id4*, and *Nanos2*; Shinohara *et al*. 1999, Kubota *et al*. 2003, Buaas *et al*. 2004, Costoya *et al*. 2004, Kanatsu-Shinohara *et al*. 2004, Ohmura *et al*. 2004, Buageaw *et al*. 2005, Seandel *et al*. 2007, Tokuda *et al*. 2007, Zheng *et al*. 2009, Oatley *et al*. 2011, Sada *et al*. 2012). Notably, the proteins identified generally mark (undifferentiated) spermatogonia and are by no means specific for SSCs in rodents. It is also applicable to their domestic counterparts. Yet, Inhibitor of DNA binding 4 (*Id4*) is recently identified as a marker for putative SSCs in mice due to its specific expression in single spermatogonia, which is undoubtedly a milestone in SSC characterization (Oatley *et al*. 2011). In comparison, the known phenotypic markers of spermatogonia in domestic animals are relatively limited. Despite the rarity, a few markers have been found to be consistently expressed in spermatogonia from domestic species (Table 1). An illustration of this point is ubiquitin carboxyl-terminal hydrolase L1 (*UCHL1*). *UCHL1*, also known as *PGP9.5*, is first shown to be expressed by mouse spermatogonia (Kon *et al*. 1999). We and others found the expression of *UCHL1* in prepubertal testes from pigs, cattle, buffalo, and goats (Luo *et al*. 2006, 2009, Herrid *et al*. 2007, Goel *et al*. 2010, Fujihara *et al*. 2011, Heidari *et al*. 2012, Zheng *et al*. 2013*a*). *UCHL1* is expressed in premeiotic male germ cells and does not show an affinity for somatic cells, which makes it an optimal marker for spermatogonia in domestic testes. Similarly, promyelocytic leukemia zinc-finger protein (PLZF, also known as ZBTB16), a transcription factor essential for the maintenance and self-renewal of SSCs, is initially found to be expressed by mouse undifferentiated spermatogonia (Buaas *et al*. 2004, Costoya *et al*. 2004). Later, its expression in a sub-population of gonocytes and SSCs/progenitors in pigs, cattle, sheep, goats, and equids is verified (Luo *et al*. 2006, Borjigin *et al*. 2010, Reding *et al*. 2010, Costa *et al*. 2012, Song *et al*. 2013, Zheng *et al*. 2013*a*).

Unlike *UCHL1* and *PLZF*, lectin *Dolichos biflorus* agglutinin (*DBA*) is not expressed in murine germ cells. Moreover, the expression of *DBA* does not show consistency in domestic species. In boars, *DBA* only marks primitive germ cells, i.e. gonocytes and primitive spermatogonia, and *DBA* binding to male germ cells is progressively lost with age (Goel *et al*. 2007). By comparison, *DBA* is demonstrated to be expressed in gonocytes and type A spermatogonia from prepubertal bovine and buffalo testes, with an expression profile similar to *UCHL1* (Ertl & Wrobel 1992, Herrid *et al*. 2007, Goel *et al*. 2010, 2011, Fujihara *et al*. 2011). Nonetheless, the expression of *DBA* is not discerned in germ cells at any stage of ovine development (Borjigin *et al*. 2010). Thus, even though *DBA* could be adopted to characterize gonocytes and spermatogonia from some large animals, this is not the case in sheep.

In addition to the aforementioned three markers typically used to identify spermatogonia in domestic animals, some markers of undifferentiated spermatogonia in murine are conserved among species. *THY1* (previously known as CD90), for example, is first utilized as a surface marker of undifferentiated spermatogonia in rodents, humans, and nonhuman primates (Kubota *et al*. 2003, Ryu *et al*. 2004, Hermann *et al*. 2009, He *et al*. 2010). Reding *et al*. and our group demonstrated the expression of *THY1* in gonocytes and undifferentiated spermatogonia from cattle and pigs (Reding *et al*. 2010, Zheng *et al*. 2013*a*) respectively. Recently, both Abbasi *et al*. (2013) and Wu *et al*. (2013) have reported the expression of *THY1* in undifferentiated spermatogonia

Table 1 Molecular markers of spermatogonia in testes of domestic animals.

Marker	References	Species	Approaches	Expressed in gonocytes or spermatogonia
UCHL1 (PGP9.5)	Luo *et al.* (2006)	Pig	IHC+ICC	Gonocytes and spermatogonia
	Herrid *et al.* (2007)	Cattle	IHC+ICC	Gonocytes and spermatogonia
	Luo *et al.* (2009)	Pig	IHC+ICC	Gonocytes and spermatogonia
	Goel *et al.* (2010)	Buffalo	IHC+ICC	Prepubertal spermatogonia
	Fujihara *et al.* (2011)	Cattle	IHC+ICC+WB	Gonocytes and spermatogonia
	Heidari *et al.* (2012)	Goat	IHC+ICC	Gonocytes
ZBTB16	Luo *et al.* (2006)	Pig	IHC+ICC	Gonocytes and spermatogonia
	Borjigin *et al.* (2010)	Sheep	IHC+ICC	Gonocytes and spermatogonia
	Reding *et al.* (2010)	Cattle	IHC+ICC+qPCR+WB	Gonocytes and spermatogonia
	Song *et al.* (2013)	Goat	IHC+ICC+qPCR	Gonocytes and spermatogonia
	Costa *et al.* (2012)	Equid	IHC+WB	Spermatogonia
DBA	Goel *et al.* (2007)	Pig	IHC+ICC	Gonocytes (staining weak/absent in spermatogonia)
	Ertl & Wrobel (1992)	Cattle	IHC	Gonocytes and spermatogonia
	Herrid *et al.* (2007)	Cattle	IHC+ICC	Gonocytes and spermatogonia
	Goel *et al.* (2010)	Buffalo	IHC+ICC	Prepubertal spermatogonia
	Fujihara *et al.* (2011)	Cattle	IHC+ICC+XenoTP	Gonocytes and spermatogonia
	Borjigin *et al.* (2010)	Sheep	IHC	No expression detected
THY1 (CD90)	Reding *et al.* (2010)	Cattle	FACS+MACS+IHC+ICC+qPCR+WB+XenoTP	Gonocytes and spermatogonia
	Zheng *et al.* (2013*a*)	Pig	MACS+IHC+ICC+qPCR+WB	Gonocytes and spermatogonia
	Abbasi *et al.* (2013)	Goat	MACS+IHC+ICC+qPCR+WB+XenoTP	Spermatogonia
	Wu *et al.* (2013)	Goat	MACS+IHC+ICC+qPCR	Spermatogonia
GFRα1	Lee *et al.* (2013)	Pig	MACS+IHC+ICC	Gonocytes
	Costa *et al.* (2012)	Equid	IHC+WB	Spermatogonia
NANOG2	Goel *et al.* (2008)	Pig	IHC	Gonocytes in neonatal testes, differentiated germ cells, and undifferentiated spermatogonia (rare) in prepubertal testes
	Fujihara *et al.* (2011)	Cattle	IHC+ICC+WB	Gonocytes in neonatal testes, differentiated spermatic cells, and spermatogonia (rare) in adult testes
POU5F1	Goel *et al.* (2008)	Pig	IHC	Differentiated germ cells and undifferentiated spermatogonia (rare) in prepubertal testes
	Goel *et al.* (2010)	Buffalo	IHC+ICC	Spermatogonia
	Fujihara *et al.* (2011)	Cattle	IHC+ICC+WB	Gonocytes in neonatal testes, differentiated spermatic cells, and spermatogonia (rare) in adult testes
	Mahla *et al.* (2012)	Buffalo	IHC+ICC+WB	Gonocytes and spermatogonia in prepubertal testes, round spermatids in adult testes
SSEA1	Kim *et al.* (2013)	Pig	FACS+IHC+ICC+XenoTP	Spermatogonia
Claudin-8	McMillan *et al.* (2013)	Cattle	FACS+MACS+IHC+ICC+qPCR	Spermatogonia
CSF1R	Costa *et al.* (2012)	Equid	IHC+WB	Spermatogonia

IHC, immunohistochemistry; ICC, immunocytochemistry; WB, western blot; qPCR, quantitative real-time PCR; XenoTP, xenotransplantation; FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting.

from goat testes, further corroborating that *THY1* is a conserved surface marker among species. GDNF family receptor α1 (*Gfrα1*) has long been proposed as a surface marker for undifferentiated spermatogonia in mouse testes (Buageaw *et al.* 2005), and recently its expression in a sub-population of gonocytes in neonatal porcine testes has been reported (Lee *et al.* 2013). Not surprisingly, undifferentiated spermatogonia with SSC potential are shown to share some phenotypes with embryonic stem (ES) cells. Stage-specific embryonic antigen-1 (*SSEA1*) specifically marks a subset of undifferentiated spermatogonia in prepubertal boars (Kim *et al.* 2013), and the positive staining of Claudin-8 is discernible in putative SSCs and a handful of Sertoli cells in bovine testes (McMillan *et al.* 2013). Nevertheless, transcription factors, *NANOG* and *OCT3/4*, which are related to the pluripotency of stem cells, seem to be conserved but are less dependable markers for undifferentiated spermatogonia from domestic species, due to the dynamic changes in their expression during germ cell development (Goel *et al.* 2008, 2010, 2011, Fujihara *et al.* 2011, Mahla *et al.* 2012; Table 1).

Despite the accomplishments of spermatogonial characterization, no specific marker for SSCs is currently

available. Specifically, even though differentiating germ cells express a different set of markers, such as *c-Kit*, *Stra8*, and *Smc6* (Yoshinaga *et al*. 1991, Giuili *et al*. 2002, Verver *et al*. 2013), the known molecular markers mentioned earlier are usually expressed by SSCs and undifferentiated spermatogonia that are phenotypically identical to the stem cells, but have committed to differentiation and are thought to have lost stem cell activity to some extent. Furthermore, some markers can be expressed in somatic cells as well. As a matter of fact, germ cell transplantation is presently perceived as the only approach to distinguishing between functional stem cells and other spermatogenic subtypes. In this sense, the identification of a unique marker for SSCs, which remains a challenge for researchers in this field, will greatly facilitate the studies of SSCs. In addition, the knowledge of biological markers for SSCs/progenitors in domestic species needs to be expanded.

Isolation and enrichment of spermatogonia from domestic animals

In adult mice, only 0.02–0.03% of the total germ cells have stem cell capacity (Tegelenbosch & de Rooij 1993). Likewise, SSCs are extremely limited in testes from domestic animals. As a consequence, it is necessary to isolate and enrich SSCs with high viability and purity, for the sake of subsequent culture or manipulation of these cells. The development of characterization of spermatogonia lays the foundation for following enrichment, in that some biological markers have the potential to make a difference in this process when using fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS). Apart from the two approaches referring to the phenotype of spermatogonia, physical methodology such as differential plating, the selection with extracellular matrix (ECM), velocity sedimentation, or density gradient centrifugation equally serves as common practise to enrich spermatogonia.

Herrid *et al*. (2009*a*) utilized several approaches to obtaining enriched spermatogonia from bovine testes, and found that the combination of enrichment techniques may significantly augment the purity of spermatogonia. Uniformly, both Izadyar *et al*. (2002) and Goel *et al*. (2010) combined differential plating with Percoll centrifugation to enhance the purity of type A spermatogonia, with results of over 70 and 55% in prepubertal cattle and buffalo respectively. The highest purity (90%) of type A spermatogonia from buffalo testes is achieved by Ahmad *et al*. (2013), who adopted the selection with ECM (laminin and gelatin) followed by Percoll gradient separation. In sheep, Borjigin *et al*. (2010) evaluated the enrichment efficiency of differential plating and revealed that the maturity of testes is an important element of spermatogonia recovery. Zhu *et al*. (2012) found that fibronectin and laminin are optimum ECM for the enrichment of spermatogonia from dairy goats. In boars, even though the efficiency of purification varies with several different methods (Luo *et al*. 2006, Goel *et al*. 2007, Kim *et al*. 2010, 2013, Yang *et al*. 2010, Yang & Honaramooz 2011, Zheng *et al*. 2013*a*), neonatal gonocytes with as high as 90% of purity are obtained via Nycodenz centrifugation followed by differential plating (Yang & Honaramooz 2011).

FACS, which involves the molecular signature of cells, is exploited to enrich spermatogonia from large animals. FACS of SSEA1$^+$ fraction leads to a markedly enriched population of undifferentiated spermatogonia from pigs (Kim *et al*. 2013), whereas FACS yields a fourfold rise in DBA$^+$ cells from cattle (Herrid *et al*. 2009*a*). Like FACS, MACS can be used to enrich spermatogonia, and now it is becoming increasingly prevalent in the isolation and enrichment of spermatogonia from domestic testes. Reding *et al*. (2010) adopted MACS to enrich undifferentiated spermatogonia in prepubertal bovine testes, resulting in a THY1$^+$ fraction composed of 64.4% of PLZF$^+$ cells. Similarly, our group demonstrated that a cell population comprising 55% of UCHL1$^+$ cells could be obtained by MACS of THY1$^+$ cells from neonatal piglets (Zheng *et al*. 2013*a*). In goats, MACS of THY1$^+$ cells contributes to a significant enrichment of undifferentiated spermatogonia (Abbasi *et al*. 2013, Wu *et al*. 2013). Reports concerning the utilization of MACS and other surface markers to enrich bovine spermatogonia are also available (Herrid *et al*. 2009*a*, McMillan *et al*. 2013). Conclusively, MACS is of inherent advantage in comparison with other approaches. For one thing, MACS is not time-consuming and is easy to reproduce; therefore, this technique could be readily implemented without the requirement for abundant experience. For another, MACS does not require a multitude of cells, but does select the cells with high viability, which is important for subsequent culture or manipulation of SSCs. Also, MACS can easily be scaled to accommodate larger cell numbers of crude single-cell suspensions, while with FACS, sorting time increases dramatically with higher cell input. Given the strengths, MACS is believed to play increasingly vital roles in the isolation and enrichment of spermatogonia from domestic animals.

Of note, it is wrong to equate the enrichment of spermatogonia with that of SSCs. In other words, the enrichment is usually for (undifferentiated) spermatogonia but not necessarily for SSCs. Owing to the absence of a specific marker for SSCs, it is only when transplantation assay is performed that the enrichment of SSCs can be quantified.

In vitro culture of SSCs from domestic animals

In vitro culture of SSCs is an essential tool to expand and manipulate this rare cell population, and a long-term SSC culture is regarded as a prerequisite for *in vitro* exploration of SSCs self-renewal and differentiation.

To date, scientists have succeeded in establishing long-term cultures of SSCs from mice, rats, and hamsters (Kanatsu-Shinohara *et al*. 2003, 2008, Kubota *et al*. 2004, Ryu *et al*. 2005). By contrast, even though researchers have made strenuous efforts to culture SSCs from domestic animals, the propagation of SSCs *in vitro* could only be maintained for a short time (no longer than 2 months; Aponte *et al*. 2006, 2008, Luo *et al*. 2006, Goel *et al*. 2007, 2009, 2010, Kuijk *et al*. 2009, Xie *et al*. 2010, Fujihara *et al*. 2011, Bahadorani *et al*. 2012, Heidari *et al*. 2012, Kala *et al*. 2012, Nasiri *et al*. 2012, Zhu *et al*. 2012, Kadam *et al*. 2013, Zheng *et al*. 2013*b*). As shown in relevant reports, putative SSCs in domestic testes can be isolated and primary cultured with ease, but the proliferation of putative SSCs experiences a gradual decrease during sub-culture, and over time, differentiation and apoptosis dominate the cellular events, with the final consequence that the propagation of SSCs comes to a standstill. SSCs from large animals may hold unique characteristics, since a long-term culture system developed for rodent SSCs could not support the persistent propagation of SSCs from domestic species. It is not unrealistic, however, to establish a long-term culture of SSCs from domestic animals in the near future. To fulfill this goal, three issues need to be taken into account.

First, the culture medium is of overriding importance to the long-term cultivation of SSCs. At present DMEM (high glucose) and DMEM/F12 are the most widely used media in cultures of SSCs from domestic animals (Luo *et al*. 2006, Goel *et al*. 2007, 2009, 2010, Xie *et al*. 2010, Fujihara *et al*. 2011, Bahadorani *et al*. 2012, Heidari *et al*. 2012, Kala *et al*. 2012, Nasiri *et al*. 2012, Zhu *et al*. 2012, Kadam *et al*. 2013, Zheng *et al*. 2013*b*), probably due to the availability and affordability of the media. The corresponding studies, on the other hand, show that DMEM, in all likelihood, promotes both self-renewal and differentiation of SSCs. StemPro medium, a proprietary product likely to contain unexposed ingredients, is adopted in long-term cultures of SSCs from mice and hamsters (Kanatsu-Shinohara *et al*. 2003, 2008). Aponte *et al*. (2008) and Kuijk *et al*. (2009) reported the application of StemPro medium to *in vitro* propagation of SSCs from bulls and pigs respectively. However, neither of the aforementioned two media results in the long-term maintenance of SSCs from domestic animals, which leaves room for further improvement in this respect.

Second, it is imperative that appropriate growth factors are included in the culture medium. There is no controversy regarding the use of GDNF for long-term maintenance and self-renewal of murine SSCs during culture. Recently Lee *et al*. (2013) have reported that *Gfrα1*, one of the GDNF family of receptors, is expressed on the surface of gonocytes in boars, showing that GDNF may also play indispensable roles in long-term cultures of SSCs from domestic species. Nevertheless, when culturing SSCs from large animals, the functions of other usually utilized growth factors in rodent SSC culture, such as bFGF, epidermal growth factor (EGF), leukemia inhibitory factor (LIF), and CSF1, if any, remain largely elusive. Indeed, there are inconsistencies in the reports describing the effects of growth factors on the propagation of SSCs from domestic animals (Aponte *et al*. 2006, 2008, Kuijk *et al*. 2009, Zhu *et al*. 2012, Kadam *et al*. 2013, Zheng *et al*. 2013*b*). Even though the conflicting results can sometimes be ascribed to the differences in species or ages of the donors, the ratio of SSCs to contaminating somatic cells at the outset of culture, and even to distinct batches or manufacturers of the growth factors, one fact related to this issue cannot be neglected: it is somewhat imprecise to quantify the cultured SSCs by approaches other than transplantation assay. Currently two approaches are popular in the quantification of the cultured domestic SSCs: SSC-derived colony counts and putative SSC (individual cell positive for SSC markers) counts. Both approaches are accompanied by drawbacks. SSC-derived colonies, albeit morphologically distinct from ES-like colonies, are usually aggregates of germ cells and the surrounding somatic cells, and it can be difficult to distinguish some colonies as they are easily covered up by the overwhelming somatic cells. As for the latter, due to the absence of a specific marker for SSCs, positive staining cells at most represent undifferentiated spermatogonia rather than bona fide SSCs, and there is research showing that cultured somatic cells are also able to react positively to a few (undifferentiated) spermatogonia markers (e.g. *Uchl1* and *Nanog2*; Luo *et al*. 2006, Goel *et al*. 2009). In a sense, when there is no transplantation performed as a functional assay (Fig. 1), nothing can be said about SSCs. Besides, studies of mechanisms for self-renewal and differentiation of SSCs from domestic species are currently not available. Hence, when propagating SSCs from large animals *in vitro*, their requirements for specific growth factors as well as their molecular mechanisms for the regulation of self-renewal and differentiation need to be further probed.

Last but not least, serum and feeders are integral parts of a long-term culture of SSCs. Although a feeder- and serum-free culture system for mouse SSCs was established recently (Kanatsu-Shinohara *et al*. 2011), it is not feasible to cultivate SSCs from domestic animals with neither serum nor feeders. It is noticeable that serum contains undefined components and tends to act as a catalyst for overwhelming somatic cells. In goats and pigs, high concentration of serum has been shown to have a detrimental influence on SSC proliferation (Bahadorani *et al*. 2012, Zheng *et al*. 2013*b*). Therefore, it is sensible to reduce serum in SSC culture. Given the choice, it would be stimulating as well to test alternatives for serum such as knockout serum replacement and B27 supplement, which are artificial and have certain components. In terms of feeders, autologous Sertoli cells

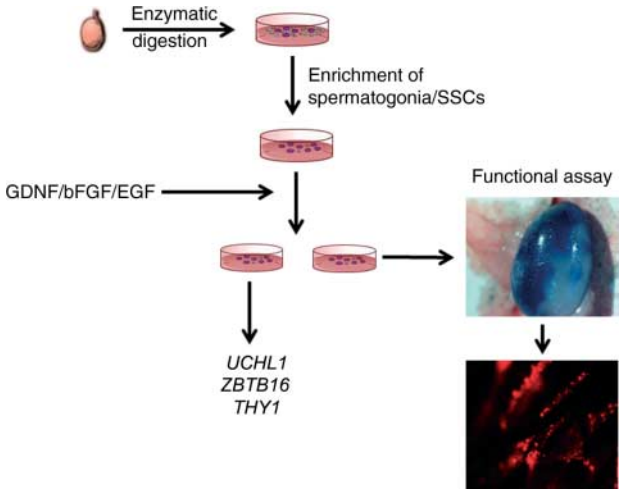

Figure 1 A schematic representation of procedures involved in enrichment and culture of spermatogonia/SSCs from domestic animals. Donor testis tissue is collected and enzymatically digested to get a single-cell suspension. Spermatogonia/SSCs can be enriched by way of FACS, MACS, or physical methodology (such as differential plating, velocity sedimentation, or density gradient centrifugation). The enriched spermatogonia/SSCs undergo *in vitro* propagation in the presence of growth factors (such as GDNF, bFGF, and EGF). The spermatogonia/SSC identity of the cultured cells is verified by the expression of molecular markers (such as *UCHL1*, *ZBTB16*, and *THY1*) or functional assay. SSCs can survive and colonize in seminiferous tubules of the recipient mouse testes.

are typically used in cultures of SSCs from large species. One drawback of this option is that Sertoli cells are capable of secreting a variety of growth factors, including those committed to SSC differentiation. Nasiri *et al*. concluded SIM mouse embryo-derived thioguanine and ouabain resistant (STO) that is exploited in long-term cultures of mouse and rat SSCs as a fitting feeder layer to multiply bovine spermatogonia *in vitro* (Kubota *et al*. 2004, Ryu *et al*. 2005, Nasiri *et al*. 2012). Combing this report with the study which described the substitution of laminin for feeder cells during *in vitro* propagation of human SSCs (Sadri-Ardekani *et al*. 2009), it might be preferable to use STO or laminin for long-term cultures of SSCs from domestic testes.

Transplantation of SSCs from domestic animals

In 1994, Brinster & Zimmermann (1994) established germ cell transplantation. Since then, this functional assay radically changed the study of SSCs and spermatogenesis because this technique genuinely identifies SSCs *in vivo*: functional stem cells from donor testes could relocate to the basement membrane provided that they are transplanted into the seminiferous tubules of a host. This report and subsequent studies verified that SSCs from mice or rats could reinitiate spermatogenesis and generate functional sperm after transplantation into recipient mouse testes (Brinster & Zimmermann 1994, Jiang & Short 1995, Ogawa *et al*. 1999). The spectacular discoveries impel scientists to investigate the potential of SSCs from large animals in this respect. As reported, SSCs from domestic species could colonize and proliferate in host mouse testes after transplantation, but could not give rise to entire spermatogenesis (Dobrinski *et al*. 1999, 2000, Izadyar *et al*. 2002, Oatley *et al*. 2002, Kim *et al*. 2006; Table 2), probably due to the phylogenetic disparity between donors and hosts. Later, allogeneic transplantation of SSCs was tested in boars, bulls, goats, sheep, and dogs (Honaramooz *et al*. 2002*a*, 2003, Izadyar *et al*. 2003, Herrid *et al*. 2006, 2009*b*, Kim *et al*. 2008), and the development of the donor germ cells was observed in those recipients of homogeneous species (Fig. 2A and Table 2).

The desirable testicular xenografting has been developed to become an alternative for germ cell transplantation over the last decade. A breakthrough in this regard was achieved in 2002. Technically, testicular tissue from neonatal piglets and goats was introduced into a host (an immuno-deficient mouse), and viable sperm was attained in this way (Honaramooz *et al*. 2002*b*; Fig. 2B). From that point onwards, xenografting of testicular tissue from a wide array of domestic animals

Table 2 Summary of germ cell transplantation in different donors of domestic animals and recipient species.

Donor	Recipient	Reference	Colonization of SSCs	Donor-derived spermatozoa	Offspring
Pig	Mouse	Dobrinski *et al*. (2000)	+	−	−
Pig	Pig	Honaramooz *et al*. (2002*a*)	+	+	−
Cattle	Mouse	Dobrinski *et al*. (2000)	+	−	−
Cattle	Mouse	Oatley *et al*. (2002)	+	−	−
Cattle	Mouse	Izadyar *et al*. (2002)	+	−	−
Cattle	Cattle	Izadyar *et al*. (2003)	+	+	−
Cattle	Cattle	Herrid *et al*. (2006)	+	+	−
Goat	Goat	Honaramooz *et al*. (2003)	+	+	+
Sheep	Sheep	Herrid *et al*. (2009*b*)	+	+	+
Horse	Mouse	Dobrinski *et al*. (2000)	+	−	−
Rabbit	Mouse	Dobrinski *et al*. (1999)	+	−	−
Cat	Mouse	Kim *et al*. (2006)	+	−	−
Dog	Mouse	Dobrinski *et al*. (1999)	+	−	−
Dog	Dog	Kim *et al*. (2008)	+	+	−

+, Positive results; −, negative results.

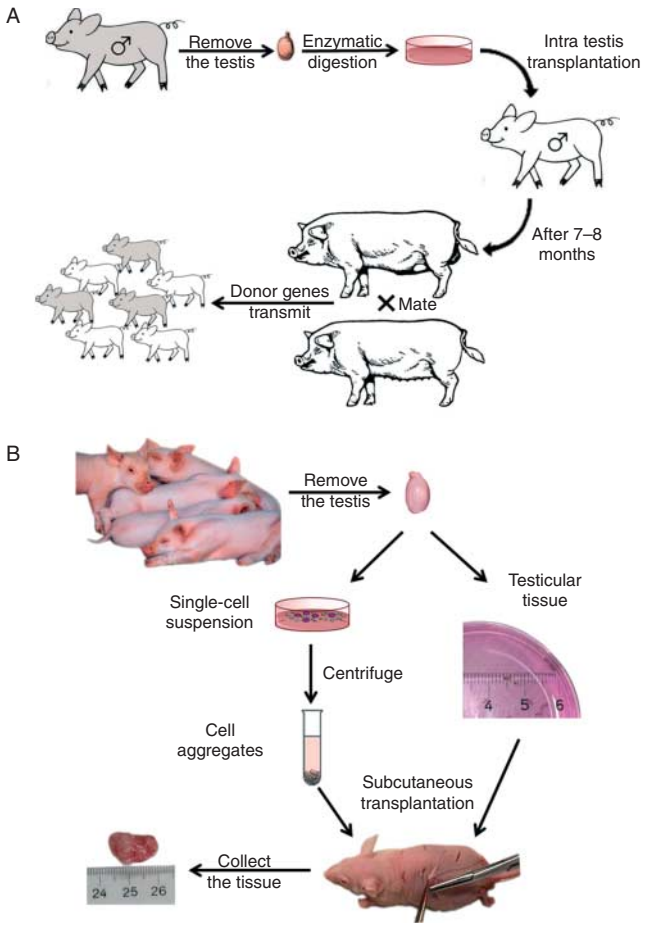

Figure 2 A schematic representation of procedures involved in transplantation of SSCs from domestic species. (A) Donor testis tissue is collected and enzymatically digested to get a single-cell suspension. Before SSC transplantation, the recipient animals are treated with busulfan or suffer local irradiation of the testes to curtail endogenous SSCs. The transplanted SSCs can form colonies of donor-derived spermatogenesis and produce spermatozoa so as to allow the recipient to sire progeny carrying the donor genes. (B) Testis tissue from donor domestic animals can be cut into small pieces and grafted into the back skin of immuno-deficient mouse. Alternatively, donor testis tissue can be digested into a single-cell suspension. The single-cell suspension is aggregated and transplanted into the back skin of immuno-deficient mouse. The grafted testis tissue or cell aggregates survive and grow up, and even produce donor-derived spermatozoa in the recipient mouse.

including bulls, sheep, horses, and cats has been reported (Oatley *et al*. 2004, Rathi *et al*. 2006, Zeng *et al*. 2006, Kim *et al*. 2007), with the full spermatogenesis achieved by this means. In 2010, viable piglets were first produced with sperm arising from testicular xenografting (Nakai *et al*. 2010).

A blockbuster with regard to *in vivo* study of SSCs might be the emergence of a mighty technique named *de novo* morphogenesis of testis tissue (Honaramooz *et al*. 2007). Technically, somatic cells and primitive germ cells from neonatal porcine testes were aggregated and implanted under the dorsal skin of immuno-compromised mouse. After a 4-week period, the transplanted cells reconstructed a spermatogenic unit resembling seminiferous tubules, in which germ cells, Sertoli cells, and interstitial cells were arranged in a way akin to their counterparts in testes. After 30 weeks, elongated spermatids were discernible in the *de novo* tissue (Fig. 2B). This approach has soon been recapitulated in donor cells from rodents and sheep (Kita *et al*. 2007, Arregui *et al*. 2008). Despite that, one major barrier that needs to be removed is the low spermatogenic efficiency (10–20%) resulting from this technique (Dores *et al*. 2012).

Summary

The fulfillments of SSC characterization, culture, and manipulation in rodents prompt scientists to scrutinize the progressions of their nonrodent counterparts. This review, for this purpose, addresses the recent advances in characterization, isolation, culture, and transplantation of SSCs from domestic animals. The prospects as well as the hindrances that need to be conquered in corresponding fields are also discussed. As SSCs have been consolidating their status as a research focus in reproductive biology and are highly likely to revolutionize the reproductive technology for large animals, our contribution is expected to enable a clear perspective of SSCs in domestic animals, as well as to bridge the gap between their basic research and potential application to animal production.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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