EDITORIAL

Spermatogonial stem cells: Current biotechnological advances in reproduction and regenerative medicine

Pedro Manuel Aponte

Pedro Manuel Aponte, AGROCALIDAD/Project Prometeo - SENESCYT, Quito 170184, Ecuador
Author contributions: Aponte PM solely contributed to this paper.

Correspondence to: Pedro Manuel Aponte, DVM, PhD, Scientific Adviser at AGROCALIDAD/Project Prometeo - SENESCYT, Av. 9 de Octubre N22-48 y Jerónimo Carrión, Casa Patrimonial, Quito 170184, Ecuador. apontep@gmail.com
Telephone: +593-2-2372844
Fax: +593-2-2372844

Abstract

Spermatogonial stem cells (SSCs) are the germ stem cells of the seminiferous epithelium in the testis. Through the process of spermatogenesis, they produce sperm while concomitantly keeping their cellular pool constant through self-renewal. SSC biology offers important applications for animal reproduction and overcoming human disease through regenerative therapies. To this end, several techniques involving SSCs have been developed and will be covered in this article. SSCs convey genetic information to the next generation, a property that can be exploited for gene targeting. Additionally, SSCs can be induced to become embryonic stem cell-like pluripotent cells *in vitro*. Updates on SSC transplantation techniques with related applications, such as fertility restoration and preservation of endangered species, are also covered on this article. SSC suspensions can be transplanted to the testis of an animal and this has given the basis for SSC functional assays. This procedure has proven technically demanding in large animals and men. In parallel, testis tissue xenografting, another transplantation technique, was developed and resulted in sperm production in testis explants grafted into ectopical locations in foreign species. Since SSC culture holds a pivotal role in SSC biotechnologies, current advances are overviewed. Finally, spermatogenesis *in vitro*, already demonstrated in mice, offers great promises to cope with reproductive issues in the farm animal industry and human clinical applications.

Key words: Cell culture; Spermatogonial stem cells; Spermatogenesis; Pluripotency; Transplantation; Fertility

Core tip: This article reviews the current body of knowledge on biotechnological applications of spermatogonial stem cells (SSCs). SSCs are the founding adult germ stem cells of the sperm producing process spermatogenesis. SSCs belong to the male germline and can be expanded *in vitro* in several species. Through mechanisms not fully understood they can derive pluripotent stem cells *in vitro*. Thus, they can be genetically modified with some advantages over embryonic stem cell-based technologies. SSCs can be transplanted to homotopical or ectopical locations, offering great potentials in fertility related issues and regenerative clinical applications in domestic or wild animals and men.

Aponte PM. Spermatogonial stem cells: Current biotechnological

INTRODUCTION

Spermatogonial stem cells (SSCs), the germ stem cells of the seminiferous epithelium in the testis are the founder cells of a sperm producing stem cell system called spermatogenesis. SSCs were initially regarded as unipotential cells. Nowadays, however, current knowledge about SSCs has expanded to new limits and the general concept of SSCs as cells just supporting the production of the terminally differentiated sperm has rather radically changed. As a result of continuous and elaborate SSC biology research, the manipulation of this special cell type offers novel biotechnological solutions to contemporary human problems. SSCs are the only adult stem cells capable of transmitting the genome of a given species from one generation to the next, while at the same time having the capacity to convert into pluripotent stem cells[1-3]. They can also give rise to cells from the basic three embryonic layers *in vitro*, which opened new opportunities for regenerative medicine[1]. Since SSCs are adult cells, they do not bring about ethical concerns as occurs with the use of embryonic stem (ES) cells. However, the use of SSCs is not without risk, since they show some issues regarding epigenetic inheritance, as such a topic will be covered later on this article.

The faculty of SSCs to deliver genes over generations makes them a valuable vehicle for transgenesis techniques. This is particularly important for gene targeting in domestic animals, in which, contrary to laboratory rodent species, the techniques involving SSCs are still not feasible. In this process, SSC culture plays a central role in the exploitation of the unique properties of SSCs for biotechnological applications. Historically, the characterization of the SSC niche *in vivo* gave the basis for SSC culture conditions, especially SSC population expansion *via* self-renewal in several species, included men. A stem cell niche consists of a microenvironment with supporting cells and a corresponding signal exchange that regulates self-renewal and differentiation of the stem cells. Accordingly, SSCs are located in the seminiferous epithelium, near the basement membrane and the niche environment normally contains factors produced by neighboring somatic cells (sertoli cells, leydig cells, and peritubular myoid cells), the basement membrane and in particular the vascular network, near where SSCs are preferentially located[4]. This microenvironment in the testis provides signals to control stem cell self-renewal, differentiation, or survival, guaranteeing a normal cell kinetics in spermatogenesis[5,6].

SSC behavior *in vitro* could be further investigated thanks to the development of germ cell transplantation assays, which allow functional verification of the presence of SSCs (see the topic Germ cell transplantation). The addition of certain growth factors to the culture media, in particular glial cell line-derived neurotrophic factor (GDNF), and the availability of a functional SSC assay, allows SSC populations to be expanded during long term culture, an advance first made in rodent models.

SSC biotechnologies are being aimed to solve fertility related issues (*i.e.*, fertility restoration in oncological patients, infertility treatments and reproduction of endangered species). They also provide important developments in germplasm preservation and the emerging technology of spermatogenesis *in vitro*. Finally, several of these applications require species-specific knowledge of SSC culture conditions as well as transplantation techniques, two aspects that will be covered in this article (for a summary of the biotechnological approaches that involve the use of SSCs, see Figure 1).

SSC ISOLATION AND CULTURE

SSC isolation procedures have been described for several domestic species and humans[7-14]. SSCs are isolated by enzymatic digestion, usually involving two steps of various combinations of proteolytic enzymes (commonly, collagenases I or IV, trypsin, hyaluronidase and DNAse I). From this enzymatic digestion, a germ cell suspension is obtained that now has to go through at least one type of purification methods, but more often a combination of them (Zheng *et al*[15], 2014 summarize the current enriching methods for SSC isolation). There are two groups of purification methods, one group based on the SSC phenotype, like fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS), and the other utilizes physical methods, like differential plating, extracellular matrix selection and density gradient sedimentation[15]. Simple models for SSC isolation include the use of young animals[16], cryptorchid animals[17], vitamin A deficient animals[18], but SSC isolation becomes more complex when their source is the adult testis with full spermatogenesis. In this scenario, all differentiating germ cell types are present and SSCs represent a smaller proportion (see the topic SSC markers). Most recent articles in which SSC isolation is carried on, include one phenotype-based method and one or more physical methods. Most researchers do not use a functional assay (germ cell transplantation) to unambiguously identify the isolated germ cells as SSCs. They instead characterize the germ cell colonies arising during culture by means of immunohistochemistry or RT-PCR, aiming to identify gene products or RNA expression respectively (see the topic SSC markers). MACS is the most widely used technique nowadays. It is simple and inexpensive, but relies on specific SSC markers usually present in subsets of the SSCs, therefore sub-sampling the
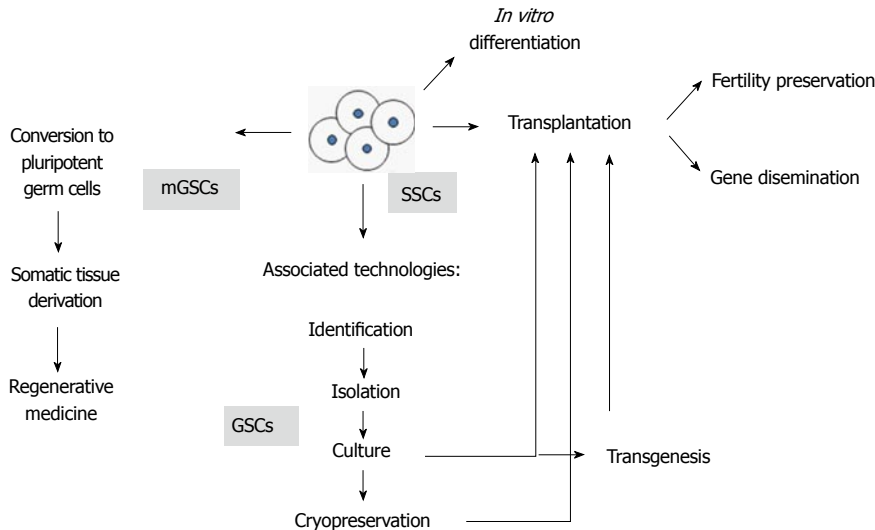
Figure 1 Overview of biotechnologies involving spermatogonial stem cells. Fertility preservation includes fertility restoration in oncological patients after therapeutic agents are administered that produce germ cell damage, strategies for infertile male patients and reproduction of endangered species. Gene dissemination represents the possibility to use several low-genetics animals to disseminate elite genes in animal production operations. Culture involves maintenance, survival, expansion and possibly genetic manipulations of spermatogonial stem cells (SSCs). Germ stem cells (GSCs), that is, SSCs that self-renew in vitro, therefore proliferating and expanding. Multipotent germ stem cells (mGSCs), which spontaneously appear in culture from within the SSC population, showing pluripotency properties[2].

general SSC population. Interestingly, one group, in a recent work, produced an enriched human SSC suspension by using MACS and the marker ITGA6 (alpha 6 integrin) together with a proper authentication of SSCs through the germ cell transplantation assay[11]. A recent novel approach for SSC isolation involved the combination of traditional isolation methods with testicular tissue grafting (see the topic Germ cell transplantation). In this work, adult mouse testis tissue was grafted into the dorsal skin of immunodeficient mice and after 2-4 wk the researchers observed the depletion of advanced differentiating germ cells of the seminiferous epithelium. Afterwards, the tissues left only with undifferentiated type A spermatogonia (a population including the SSCs) were recovered. The SSCs were isolated from the tissues, which were not only able to proliferate in vitro but also to recapitulate spermatogenesis in receptor testes devoid of endogenous spermatogenesis and furthermore to produce offspring through the original donor derived sperm[19]. This method, although perhaps not as practical as MACS, results in a larger true SSC popu-lation since the whole array of markers confirm the phenotypic profiles of SSCs.

Culture conditions for SSC propagation in vitro were first developed in mice[16,20]. This was the base for the development of domestic animals SSC culture systems[7-10,21-25]. Most of these systems have in common the use of GDNF and serum. Common growth factors, besides GDNF, include LIF, FGF2 and EGF[26], however important responses of SSCs regarding maintenance, survival and growth in vitro are dependent on age, species and even the strain of the donor animals[27]. Very recently, a feeder/serum-free culture was established for mice SSCs[28] and this method was improved for maintenance of SSCs[29].

This represents a big advance because serum, when present, introduces unknown variable factors into the culture systems[27] besides promoting the growing of contaminating somatic cells[2]. Current efforts are being made to set up functional human SSC culture systems[11,30,31].

SSC DIFFERENTIATION *IN VITRO*

Normally, SSCs self-renew during the steady state cell kinetics of spermatogenesis. They do so to maintain the undifferentiated pool while at the same time producing daughter cells that enter the differentiation path, ultimately generating sperm[32]. Thus, the production of sperm in vitro requires culture conditions favoring the pathways of sperm production and not self-renewal. Going through the meiosis step of germ cell differentiation in vitro was one of the most difficult steps for many research groups. This fact hindered the progress in this particular research area for many years. An spermatogenesis in vitro system going beyond that developmental stage and traversing spermiogenesis was achieved and mouse sperm could be generated when testicular tissue was set on the liquid/gas interphase[33]. This system included a very simple culture medium and testicular tissues came from young animals[34]. One of the main features of this culture system is the preservation of the cytoarchitecture of the testis, that is, maintaining the structure of SSC niches. The reconstitution of spermatogenesis in vitro from SSC suspensions has proven more challenging. Some groups have obtained sperm in vitro by using testicular cell suspensions from prepubertal animals but three dimensional support matrices are necessary[35,36]. Although these successful systems have been developed for sperm production

in vitro with rodent species, it is not yet foreseeable that these techniques can be translated to a clinical setting in the near future. Human SSC differentiation in culture systems is still under investigation at the present time.

The exciting possibility of producing sperm *in vitro* has large implications for farm animal industries, human fertility restoration, preservation of endangered species and many other associated technologies related to mammalian reproduction. For instance, in the cattle industry, keeping animals in large facilities would be a thing of the past when renewable SSC pools from elite bulls produce high numbers of sperm in Petri dishes at small biotechnological facilities. In many cases of human infertility it would just be enough to have one healthy SSC derived (and possibly engineered) sperm to produce offspring through assisted reproduction technologies (ARTs). Autologous SSC culture systems could be set for individuals before oncological treatments and sperm generated *in vitro* could be cryopreserved for later use to fertilize an egg and produce offspring from these patients. This way, the danger of reintroducing malignant cells back into the patient could be avoided. SSC banking could then become possible, as another mean for germplasm preservation in many species. Moreover, the cumbersome step of transplanting genetically modified SSCs to a receptor male in order to recapitulate spermatogenesis and thus obtain transgenic sperm could then be omitted. In addition, these techniques could also provide means for preserving endangered species, for instance, animal populations with very few individuals that at the moment of dying could have their SSCs collected for sperm to be produced in the laboratory.

SSC MARKERS

The characterization of SSCs is an important tool to study their presence on culture systems and during other techniques. SSCs, representing only the 0.03% of germ cells in the testis[37], and the more numerous type A spermatogonia committed to differentiate, are morphologically undistinguishable. Furthermore, there is no general consensus on the phenotypic profile of SSC as this may vary among different species[38] and there seems to be no unique SSC marker. In general, some phenotypic characteristics of SSCs have been advanced thanks to the molecular dissection of the SSC niche in the testis. Within the testis, Sertoli cells are the main cell type involved in the SSC niche[39]. The key factor GDNF, secreted by Sertoli cells, promotes SSC proliferation[40,41]. Any receptors expressed in SSCs that bind self-renewal promoting ligand molecules from the niche define their stem cell phenotype and constitute potential SSC markers[42]. That is the case of the widely accepted marker GFRα1, the receptor for GDNF in SSCs[43]. In domestic animals, besides GFRα1, another important marker for both gonocytes (perinatal germline stem cells) and SSCs is THY1[44-46].

In monkeys, SSCs express the phenotype (CD90+ DC49f+ CD117-), SSEA-4+ PLZF+[47]. Apparently, SSEA-4 seems to be conserved in humans[48]. SSCs from humans also express GPR125[49], besides GFRα1[50]. Another human SSC marker that adds to the known panel is ITGA6[51]. Recently, a new SSC gene product has been reported: PAX7, that might represent a definite common SSC marker for several species including mice, cats, dogs, pigs, bulls, monkeys and humans[52].

SSC PLURIPOTENCY

Pluripotent stem cells so far considered for regenerative medicine strategies include inner cell mass derived ES cells[53,54], somatic derived induced pluripotent stem cells (iPS cells)[55], fetal derived embryonic germ (EG) cells[56] and SSC derived multipotent germ stem (mGS) cells[57,58]. All of these cells form teratomas when injected to seminiferous tubules or subcutaneous tissue of immunocompromised mice and contribute to chimera formation when injected to a blastocyst. When SSCs are placed in culture they proliferate in the presence of GDNF. This growth factor is produced by Sertoli cells in the testis, stimulating SSCs to self-renew[41]. SSCs proliferating in culture are designated germ stem cells (GS) cells[16]. They are unipotent, genetically and epigenetically stable, do not produce teratomas and do not contribute to chimera[59]. Extraordinarily, while in culture, they can spontaneously derive multipotent germ stem cells (designated mGS cells) at a low frequency (1 out of 30 testes)[58]. They represent SSCs with pluripotency, sharing not only ES cell traits such as self-renewal properties, tumorogenicity and chimera production but also genomic and epigenetic abnormalities[2]. Pluripotent germ cells from the testis can circumvent some of the typical objections or technical limitations of other pluripotent cells (*i.e.*, ES cells and iPS cells) for their use in therapeutic purposes. These limitations include issues in cell proliferation, cell differentiation, genetic stability, allogenicity and ethical/religious issues (reviewed in[60]). Remarkably and unlike other pluripotent cells, the conversion of GS cells into mGS cells occurs on a dedifferentiation process not dependent on genetic manipulations, which opens important theoretical possibilities for these technologies to be translated into clinical applications in regenerative medicine. Nevertheless, despite all the promising traits of mGS cells, caution is required on the grounds of their genetic and epigenetic behavior. Although GS cells maintain a normal karyotype, genomic imprinting, and SSC activity during long-term culture[59], mGS cells are unstable and tend to accumulate genomic and epigenomic abnormalities[2,61]. Also, GS-like cells derived from mouse fetal germ cells had SSC activity but some of the produced offspring had abnormal genomic imprinting patterns, which were also transmitted to future generations[56]. SSC-derived pluripotent cells

are able to generate very diverse tissues while in cell culture[1,3]. For instance, differentiated tissues appear in culture from a specific subpopulation of mouse mGS cells that show a pluripotent cell phenotype (POU5F1[+]) while at the same time expressing SSC proteins (c-Kit[+])[62].

All in all, SSCs show excellent perspectives for applications in regenerative medicine. According to the "organ niche" theory, developmental failure in organogenesis caused by defective genes, can be compensated by xenogeneic pluripotent stem cell transplantation into genetically affected blastocysts[63]. The pluripotential cells would then integrate into the embryo, resulting in chimera formation with colonization of stem cells of xenogeneic origin in the organ forming niches during development. This has been demonstrated through animal models such as mouse and pigs to successfully generate functional kidneys[64] and pancreas[63,65]. SSC-derived pluripotent stem cells, with all their inherent advantages, could be used for these purposes.

Although the current advances in the generation of testicular pluripotent cells in rodents are unquestionable, similar results in the humans have been controversial. Although one work demonstrated teratoma formation and pluripotency markers expression[66] on these cells, tumors showed a relatively small volume[67]. Several other groups have produced allegedly human mGS cells as well, but these cells, although expressing several pluripotency markers, do not produce teratomas when transplanted into the skin of immunocompromised mice[68-70]. In fact, one group recently found that human testicular embryonic-like cells (allegedly mGS pluripotent cells) do not possess a transcriptome similar to that of human ES cells but rather have the phenotype of mesenchymal stem cells (MSCs), therefore, concluding that these cells are not pluripotent and most likely not of germ cell origin but instead mesenchymal[71]. Current knowledge of human SSC culture conditions probably is insufficient and do not emulate the mouse SSC culture environment that has allowed the generation of mGS cells in this species. For further progress to be made, the exact mechanisms of SSC reprogramming need to be addressed. For instance, we know that primordial germ cells from the fetal period convert to ES-like pluripotent cells[72] *via* the AKT signaling pathway[73]. A very recent study found that spontaneous SSC reprogramming would be caused by unstable DNA methylation with involvement of the Dmrt1-Sox2 signaling pathway[74]. However, more research is required to successfully obtain human mGS cells. This will require very specific culture conditions and the molecular dissection of SSC pluripotency, all of which should continue to be investigated.

GENE TECHNOLOGIES AND SSC

ES cell-based transgenesis technologies are well developed and routinely used in the context of mouse species. However, SSCs can be used for a more straightforward route to transgenesis. ES cells, after genetically intervened, are injected into blastocysts with the hope of successful integration and contribution to the germline, which not always can be guaranteed. By contrast, SSCs are already constituents of the germline. Some other important differences with ES cells make SSCs best suited for mutagenesis (Table 1), especially in species for which ES technologies are not feasible or physiological limitations exist like in farm animals. Additionally, unlike mice, some farm animals, primates and humans are uniparous species as a consequence of not producing large numbers of oocytes under normal physiological conditions. In addition, these species go through lengthy periods of time to attain puberty and sexual maturity. SSC genetic and epigenetic stability keep these cells committed to the germline phenotype so that they do not tend to differentiate into other lineages[59]. This trait makes their potential role for gene therapies clinically safer than other pluripotent stem cells. The use of SSCs for transgenesis in farm animals in comparison to ES cell-based technologies in mice is depicted in Figure 2.

Another alternative for knockout (KO) animal production is nuclear transfer of genetically modified somatic cells, but these cells have limited proliferation capacities, the technique shows inefficient offspring production and postnatal degenerative problems. For these reasons, cloning does not efficiently compete with ES cells or SSC technologies for gene targeting.

Due to their potentials, SSCs have been used to generate KO animals in several species. Transgenic mice generation by using SSCs was first accomplished with the use of retroviral vectors, however with low efficiency[75]. Later on, with the advent of GS cells (SSCs that proliferate *in vitro*) this cell type could be expanded *in vitro* and genetically modified through vectors (viruses or plasmids) with drug resistant genes to allow for the detection of SSCs carrying the transgene. These SSCs were transplanted to recipient mice and took over the spermatogenetic functions in the new environment to ultimately produce transgenic sperm. Transgenic heterozygous males with transgene carrying sperm were mated with females and progeny was produced, near 50% of which carried the transgene[76]. This illustrates the importance of an optimal culture system in order to expand SSC populations and thus increase the efficiency of the transgene incorporation. Soon after, sophisticated techniques added to the gene function modification toolbox such as the CRE/LoxP system for conditional gene targeting, was successfully adapted to the use with SSCs[77]. Interestingly, SSC mediated rat transgenesis was achieved in 2007[78] while parallel efforts with ES cells on this species were only successful until 2010[79]. Just recently, ES cells from domestic animals could be obtained for gene targeting goals[80]. Advances with farm animal SSCs continued in parallel. For instance, goat transgenic

Table 1 Summary comparison of characteristics of embryonic stem cells and spermatogonial stem cells in the context of transgenesis tecniques

Trait	Embryonic stem cells	SSCs
Mutagenesis technical status	Available for mice. Many genes mutated	Experimental level in mouse[75], rats[78], goats[81], pigs[82]
Source	Embryo	Adult testis
Age of derivation	Embryonic period	Postnatal period
Ethical/legal concerns	Yes	No
Efficiency		5 × to 10 × higher than with ES cells
Cell differentiation state	Undifferentiated - pluripotential	Differentiated up to germ-line
Tumorogenesis	Produce teratomas	Do not produce teratomas
Chimera formation	(+)	(-)
Germline gene transmission	Do not transmit genes from one generation to the next	Transmit genes from one generation to the next
In vitro phenotype	Colonies with tightly attached cells	GS cells loosely attached (easily dissociated with trypsin)[2]
Speed of growth	Faster	Slower
Requires other non-transfected SSCs during culture	No	Yes[2]
Karyotype	Variable - unstable[1]	Normal - stable
Epigenetics	Variable - unstable DNA methylation pattern	Normal - stable DNA methylation pattern
Offspring	Abnormal	Normal

Otherwise specified, information presented is based on[2]: [1]Often lose their germ cell potential due to trisomy[16]; [2]Spermatogonial stem cells (SSCs) that proliferate in culture (GS cells) require specific non-transfected SSC densities during the process of drug selection[118]. ES: Embryonic stem.
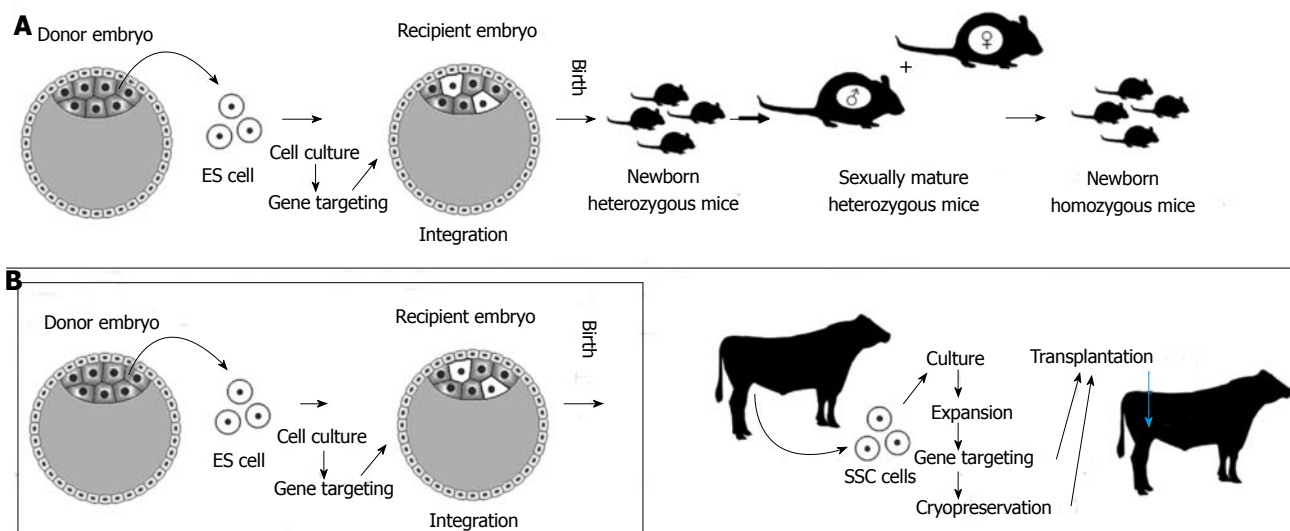

Figure 2 Alternative ways to exploit stem cells to produce transgenic animals. A: Standard method (well developed in the mouse): Embryonic stem cells (ES cells) are obtained from a donor mouse blastocyst. These cells are cultured and genetically modified (usually, DNA sequences are inserted on a gene of interest to disrupt its expression or gene knockout). ES cells are injected into another mouse blastocyst so that they can integrate into the embryo (chimera formation) with the hope that some of them will contribute to the male germline. In this case, donor germline cells will undergo homologous recombination (meiosis) in the testis after animals are born and develop towards puberty. The male animals will be heterozygous for the mutated gene and when crossed with their (also heterozygous) sisters, offspring will be obtained homozygous for the transgene; B: Transgenesis technique involving spermatogonial stem cells (SSCs), particularly useful for farm animals, for which ES cell technologies are not feasible. SSCs from postnatal animals can be expanded in culture, subject to gene targeting and transplanted to a recipient foreign testis. This way, steps used with ES cell technologies become redundant (Figure 2B, inside the box). With the advent of spermatogenesis in vitro, even the SSC transplantation procedure will not be necessary. Cryopreservation techniques for SSCs are already available.

sperm for human growth hormone was derived from male goats that had been transplanted with SSCs previously manipulated *in vitro*[81]. In a report from the same group, pig embryos, transgenic for a reporter gene (enhanced green fluorescent protein), could be obtained after oocytes were fertilized with transgenic sperm from culture transduced and later transplanted SSCs[82]. Very recently, bovine gonocytes were transduced through a lentivirus vector and the reporter transgene product proved the success of the procedure[83]. In dogs, a species with a strong potential to provide new models for human genetic diseases, SSCs have been characterized, cultured, genetically modified and allogeneically transplanted with eventual collection of transgenic sperm[84].

GERM CELL TRANSPLANTATION

SSC transplantation is an important tool for several other biotechnological applications such as gene
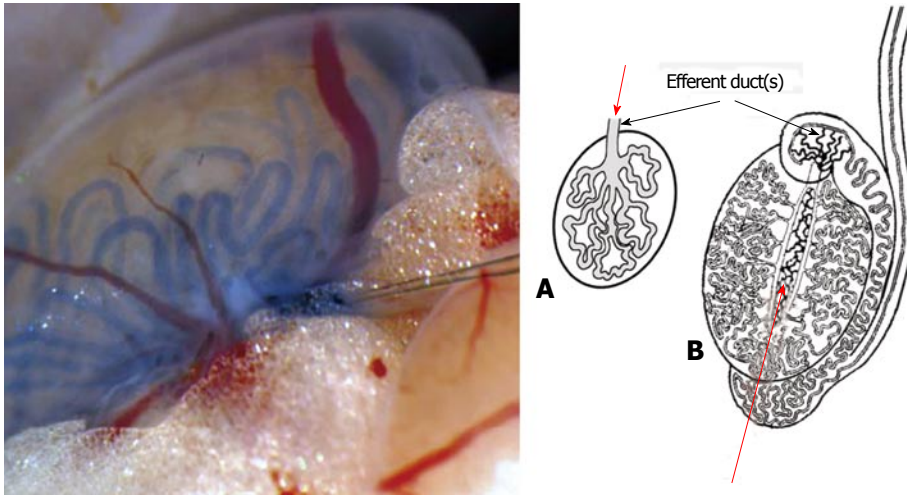
Figure 3 Species differences on anatomical points for spermatogonial stem cell injection for transplantation. The transplantation procedure for spermatogonial stem cells (SSCs) is simple in the mouse. In this species, (A) there is a single efferent duct emerging from the rete testis, which is easy to cannulate to inject SSCs (arrow). In most farm animals and men (B) several efferent ducts emerge from the testis, a reason why transplantation of SSCs is often done by injection of SSCs into the rete testis (arrow) with ultrasound guidance.

therapy or fertility restoration. The first SSC successful transplantation was reported in 1994 in the pioneering work of the Brinster's group[85]. Inspired on original hemopoietic fundamental transplantation work[86], this research group isolated germ cells from a donor mice that were later unequivocally identified as SSCs on a recipient mouse testis after injection into the seminiferous tubule system, based on their property to regenerate spermatogenesis in the foreign testicular niche (for a comprehensive review on this technique see[87]). This allogeneic type of transplantation is presently the basis for the functional assay to detect true SSCs in experimental models. The technique could be developed due to the presence of a single, easy to cannulate efferent duct in mouse species and to the fact that the mice strain used was immunocompromised. Unfortunately, this SSC transplantation procedure is technically demanding in species with several efferent ducts because, in these species they tend to be short, thin and highly convoluted (Figure 3). This anatomical condition corresponds to most farm animals[88], primates[89] and humans[90].

Xenogeneic transplantation of SSC cell suspensions into the mouse testis has been attempted by several groups[91]. For the first time, SSCs from a foreign species (rat) not only were able to colonize mouse seminiferous tubules but also to differentiate and produce normal rat sperm[92]. However, attempts with other SSC donor species showed that the farther apart in phylogenetical distance is the donor species from the mouse (recipient species), the lower level of progress of differentiation in spermatogenesis from the transplanted SSCs will be achieved within the foreign seminiferous epithelium environment (for details on SSC transplantation into the mouse testis, involving different donor species, see[91]). Accordingly, domestic animals and human beings as SSC donors are among

those species in which this technique is not yet successful. Thus, on these and many other species, functional assays to unequivocally detect the presence of their SSCs are very necessary.

Allogeneic SSC transplantation (SSCs transplanted from a donor animal to a recipient testis of another animal, both from the same species) has proven difficult in species other than rodents, particularly in large animals. Besides anatomical difficulties, each species seems to require specific technical protocols. Allogeneic transplantation has been attempted in several farm animals, including pigs, goats and bulls[93-95]. For instance, transgenic sperm from a donor goat could be unequivocally identified in a recipient buck semen, meaning that transplanted SSCs could colonize and regenerate the spermatogenetic process in the unrelated testis[96]. When the limitations of these techniques are overcome they will facilitate several of the biotechnological applications described on this article. One aspect that still needs to be addressed is the method for depletion of endogenous spermatogenesis in recipient animals. This is typically performed by using either chemical compounds like the alkylating agent busulfan or alternatively testis irradiation. However, protocols need to be species optimized, which requires further research[97,98]. SSC transplantation could also be used for gene delivering purposes in order to increase the frequency of certain genes in animal populations. This would be particularly useful in extensive systems of livestock production, in which animals reproduce through natural service. Surrogate males could be transplanted with SSCs that over-express certain genes linked to increased production traits or from elite bulls in order to disseminate the desired genes in a population. Endangered species could also be benefited with such technologies. For instance, SSCs from the wild species to be preserved could be transferred to a

phylogenetically related domestic animal embryo and this way become incorporated into the germline to eventually produce exotic sperm[99,100], this way saving the endangered germplasm.

As an alternative to SSC transplantation into testicular environments, a new technique was developed in which whole testicular tissue explants (containing SSCs attached to their niche) from the desired species are placed in ectopical places such as the back skin (subcutaneous tissue) of immunocompromised mice. This approach was first successfully tried with mouse testis tissue (donor) grafted into mouse skin (recipient)[101,102]. This technique was designated testis tissue xenografting. When xenotransplanting testicular tissues from prepubertal animals (with only undifferentiated germ cells, including SSCs), complete spermatogenesis developed within the transplanted testicular tissues under the sponsoring of the host (mouse) vascular environment and intrinsic steroidogenesis of the donor tissue. This technique has been so far successful with a wide range of donor species including hamsters, rabbits, dogs, cats, sheep, goats, horses, bovines, alpacas and monkeys[103]. Work has also shown that testicular tissue pieces can be cryopreserved and later used with this technique in various species[104,105].

Tissue xenografting has represented a step forward in SSC biology that circumvents many of the current limitations the transplantation of germ cell suspensions into the seminiferous tubules of foreign recipient animals. This technique has been privileged by researchers during the recent years because of the positive outcomes in a range of very dissimilar species. Recently, a related technique was developed in which no whole tissue pieces are transplanted into the mouse skin but instead an assorted cell suspension composed of germ cells and testicular somatic cells, including Sertoli cells. Interestingly, all these cells reassemble under the mouse skin and testicular tissue becomes organized in a process that has been called *de novo* testicular morphogenesis[106,107]. This novel technique has been tested with testicular cell suspensions from rats[108], mice[109], pigs[110], sheep[111] and recently from a wild pig, the peccary[112]. Testis xenografts have produced so far fertile sperm in pigs and the related peccaries[112-114] demonstrating that SSCs present in the testicular tissues show a correct functioning that leads to the production of viable normal sperm within the body of a foreign species.

FERTILITY RESTORATION

Infertility is one of the critical side-effects of oncologycal treatments in humans and particularly in children, an effect which is caused either by chemotherapeutic agents or irradiation. A proposed solution for this problem is the restoration of fertility *via* SSC transplantation. Since many of the patients in this situation are prepubertal (*i.e.*, do not yet produce

sperm), a current approach consists on banking testicular tissue or cell suspensions prior to the oncological treatment and submitting the patient to an autologous transplantation of the preserved material if fertility problems arise later in life[115] (for recent advances in the methods for preservation of fertility on young male human oncological patients see the comprehensive review from Goossens *et al*[116], 2013). A word of caution is valid regarding safety issues when malignant diseases are managed with transplantation procedures. Invariably, cells suspensions including SSCs to be transplanted should be devoid of cancer cells. In experiments with rats, only as few as 20 leukemia cancer cells were enough to produce the disease when transplanted into healthy rat testis[117]. It is unknown to what extent this is reproducible in humans, but as an obvious measure, cell suspensions should be screened and positively selected for SSCs and/or negatively selected for cancer cells, through approaches such as FACS or special culture conditions[116]. An alternative approach would be to use testis xenografting or allografting with the hope to restore the spermatogenic process ectopically to a degree just enough to produce a few sperm to be later used with ARTs. Nevertheless, although already developed for several animal species (see the topic on Germ cell transplantation above) testicular xenografting requires further investigations to be implemented in humans. In general, more research in monkeys and humans is still needed, particularly on safety issues.

FINAL REMARKS

The tremendous progress in SSC research during the last decade is undeniable. Appropriate conditions for SSC long-term culture are already widely known for the most important experimental model: the mouse, but research efforts are still being made to develop culture systems suitable for humans and other species, altogether with the study of the very specific SSC nutrient requirements. Advances such as SSC *in vitro* mutagenesis are already possible in the mouse. The imminent unraveling of the molecular mechanisms of SSC pluripotency will bring about many other interesting biotechnological possibilities. The long-awaited accomplishment of SSC transplantation techniques for farm animals and humans is under current development, despite the widespread use of testis xenografting in many species with high rates of success. Finally, spermatogenesis *in vitro* is already possible for laboratory species, so important applications in regenerative medicine and animal reproduction are expected.

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