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Spermatogonial stem cells: A tool to preserve and enhance fertility of diversified male species

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Abstract
Differentiation of germ cells in the testes originates from a constantly renewed small pool of stem cells which give rise to the first differentiated spermatogonia. Spermatogonia are the only cells of the germ line, which proliferate in adulthood and offer interesting applications as they are potentially totipotent and immortal cells. They are committed for gene transfer to next generations via the process of fertilization of ovum by the spermatozoa, which is sequel of a sequence of events called spermatogenesis. DNA duplication and mitotic divisions always inherit risk for DNA damage, the pool of spermatogonial stem cells thus present an entry point for changes to the integrity of the genome and offer a pathway for genetic engineering of the male gem line. Spermatogonial stem cell transplantation from a fertile donor male into the somniferous tubules of infertile recipients can result in donor-derived sperm production. The application of this method in farm animals and humans has been the subject of increasing number of studies, mostly because of its potential as an alternative strategy in producing transgenic livestock with higher efficiency and less time; pertinently it will pave the way and ray of hope for individuals having infertility especially with cancer patients besides fertility preservation.

Keywords: Spermatogonial Stem Cells, Gonocyte, Animal reproduction, Expressed enhanced green fluorescent protein

1. Introduction
Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and male fertility. SSCs are defined like all other stem cells, by their ability to balance self-renewing divisions and differentiating divisions. This balance maintains the stem cell pool and meets the proliferative demand of the testes to produce millions of sperms each day. As members of the germ line, they are potentially immortal and totipotent which might enable to use them as precursors for pluripotent stem cells. For these reasons and due to the latest methodological breakthroughs, spermatogonia are stem cells with a great perspective [1].
The presence of a stem cell population, the base for continual sperm production in the testes and the first successful SSC transplantation in mice resulting in donor-derived spermatogenesis was documented [2]. Transplantation of a mixed population of germ cells that contain SSCs into the testes of a sterile mouse restores fertility, although the genetics of the offspring will be of the donor [3]. Restoration of fertility following SSC transplantation in rodents suggests therapeutic potential for the technique in farm animals. Therapeutic uses of other adult stem cells such as hematopoietic stem cells (HSCs) motivate research to determine the basic mechanisms regulating the stem cells and factors that contribute to the stem cell niche [4, 5]. Similarly, identification of the downstream cellular signaling in SSCs following treatment with regulatory factors such as glial cell line-derived neurotropic factor (GDNF) is being intensely studied because it will impact the ability to maintain SSCs *in vitro* for therapeutic purposes [6, 7]. Likewise, investigation of factors regulating SSCs in species besides mice suggests that comparative similarities will accelerate the application of SSC in both human health and farm animal species [8-10].

2. Origin of spermatogonial stem cells
SSCs arise from gonocytes in the postnatal testes, which arise from primordial germ cells (PGCs) during foetal development. PGCs are transient cells that are first observed as small

clusters of alkaline phosphatase-positive cells in the epiblast stage embryo [11]. During the formation of the allantoin, the PGCs are passively swept out of the embryo before they start migrating via the hindgut to arrive at the indifferent gonad. PGCs replicate during the migratory phase and approximately 3000 PGCs colonize the genital ridges [12]. In the male gonad at about 13.5 days post coitus, PGCs give rise to gonocytes, which become enclosed in testicular cords formed by sertoli precursor cells and peritubular myoid cells. Gonocyte is a general term that can be subcategorized into mitotic M-prospermatogonia, T_1-prospermatogonia and T_2-propspermatogonia [13]. M-prospermatogonia is located in the centre of the testicular cords, away from the basal membrane and continues proliferating until they become T_1-prospermatogonia and enter G_0 mitotic arrest [14, 15]. Gonocytes resume proliferation during the first week after birth concomitant with migration to the seminiferous tubules basement membrane [16]. T_2-prospermatogonia that colonize the basement membrane give rise to the first round of spermatogenesis as well as establish the initial pool of SSCs that maintain spermatogenesis throughout post pubertal life [13, 17].

3. Isolation, culture and identification of spermatogonial stem cells

Procedures for isolation of testes cells vary among laboratories, depending upon the target cell types and donor species. Two step enzymatic digestions have been applied to isolate both gonocytes and SSCs in many species. In the first digestion step, Collagenase and Hyaluronidase enzymes are usually added to remove testes interstitial cells. As a second step, trypsin-EDTA (with or without additional enzymes) is used to break down seminiferous tubules, while DNase is added to prevent cellular aggregation [18]. Using two step techniques usually results in a maximum of 10% SSCs in the freshly isolated testes cells [19-21].

Recently a novel three step strategy (combining vortexing and digestion) to isolate porcine testes cells with a gonocyte proportion of more than 40% have been developed [22]. Obtaining a pure culture of spermatogonia is considered difficult owing to problems associated with a small subset of cells. Isolation of type A spermatogonia can be achieved with a purity of greater than 95% by sedimentation velocity at unit gravity [23]. As this isolation technique selects cells on the basis of their size and shape, it can only be applied to immature animals in which the number of other germ cells is less and presents a specific feature of spermatogonia. Isolation of adult spermatogonia is more difficult than isolation from immature testes because of the presence of many germ cells with similar features (the spermatogonial population accounts for < 4% of all spermatogenic cells). Magnetic cell sorting is a fast and effective method for the isolation of spermatogonia from testicular cell suspension of various species using c-kit antibodies to detect the receptor in the membrane of spermatogonia [24]. The advantage of this technique over other methods is that it can be used for the isolation of spermatogonia from adult testes. Since the isolation efficiency depends on the efficient binding of the antibody and is therefore not hampered by the presence of other germ cell types of similar shape and size. The c-kit antibody can be used as a marker to enrich differentiating spermatogonia, but probably not the 'true' stem cell. However, the description of new markers i.e. α_6 and β_1-integrin enables the selective enrichment of stem cells and other spermatogonial subtypes [25].

Initiation of spermatogonial proliferation has been observed in organ culture [26-28]. Progression of spermatogonia into meiosis has been observed in seminiferous tubule fragments [29] but not in cell [30] and co-cultures [31, 32]. Maturation from stem cells to the beginning of meiotic prophase has not been achieved *in vitro* and thus the relevance of studies *in vitro* to events *in vivo* has been questioned. It appears that both the initiation of spermatogonial differentiation from stem cells and the entry of differentiating spermatogonia into meiosis are blocked in cultured cells, even though spermatogonial stem cells are maintained after several months of culture and their transplantation re-initiates spermatogenesis [33]. However, cultures of differentiating spermatogonia allow the study of certain factors and mechanisms of spermatogonial survival that promotes clonal outgrowth and induction of apoptosis. Whether the passage of spermatogonia into meiosis (and hence cell migration through the seminiferous epithelium) depends on the structural support of the seminiferous epithelium, signaling through intercellular matrix or the availability of specific factors in the testicular microenvironment that modulate the event is yet to be explored. As yet, organ and tubule culture are the only tools for studying the factors and mechanisms involved in full pre-meiotic germ cell development until meiotic progression of cells.

Positive identification of SSCs is required to enrich their population so as to improve male germ cell transplantation. It is not possible to identify SSCs in the conglomerate of cells belonging to different stages of spermatogenic cycle merely on the basis of morphology alone. Thus it is necessary to use cell surface molecular markers expressed on spermatogonial stem cells, such as Oct-4 and Stra-8 [34]. These cell surface markers are common to other somatic stem cells, therefore the identification of germ line-specific markers is critical to characterize germ stem cells.

4. Spermatogonial stem cell preservation

Requirement of high number of germ cells from testes of donor farm animals for same day transplantation is time consuming and it is a management challenge. Along with this high volume of the tissue and presence of dense connective tissue make the process of digestion difficult, often requiring the tissue or cells to be stored overnight before germ cell transplantation [35-36]. If the time required for enrichment of germ cells followed by their transgenesis is added, the preparation time could be longer, and the need for preservation of the tissue/cells is even greater.

Cryopreservation of isolated germ cells allows their storage for extended periods of time; however, it can also damage cells, as cryopreservation of bovine germ cells resulted in cell survival rates of 50–70% [37]. The feasibility of cryopreserved fragments of porcine testicular tissue resulted in post digestion cell survival of 55–88% while maintaining the *in vivo* developmental potential [38].

Short-term preservation of testicular cells and tissues on the other hand is necessary and could be more suitable for immediate applications such as in germ cell transplantation and for shipment of cells/tissue between collaborating laboratories. Hypothermic temperatures (above freezing point but below the body temperature) causes decrease in cellular metabolism rate, oxygen demand and energy consumption leading to prolongation of cell viability. Ice-cold storage of testicular tissue for 1 or 2 days is suggestive to improve donor-derived spermatogenesis after xenografting [39].

5. Spermatogonial stem cell transplantation

The first successful description of germ cell transplantation has been reported in mice [2]. Donor spermatogenesis, recognized by developing germ cells carrying the lac-Z gene, was restored from spermatogonial stem cells microinjected into the seminiferous tubules of host animals [2]. The use of mice carrying the lac-Z transgene allowed the recognition of transplanted cells and their descendants in the testes of the host animals by X-gal staining, however later studies showed that most transplanted cells degenerate and disappear from the testes before the first meiotic donor germ cells appear after 1 month [40]. As an alternative to intra-tubular infusion of germ cells by microinjection, infusion into the efferent ducts as well as into the rete testis of the host mice have been used effectively [41].

Xenologous transfer of germ cells from a number of species into mouse testes showed that in closely related species a re-initiation of spermatogenesis is achieved, whereas in less related species a repopulation of stem cells up to certain degree is possible, but no differentiation of germ cells can be observed [42-44]. Although described complete absence of human spermatogonia after germ cell transfer into the mouse testes has been reported [45]. Also, baboon spermatogonial stem cells are able to repopulate the mouse testes indicating that even primate testicular stem cells survive and expand but do not differentiate in the rodent seminiferous epithelium [33]. Rat germ cells differentiate according to the kinetics and topography typical for rats showing that the germ cell genotype and not the sertoli cell dictate the developmental program [46]. Transplanted spermatogonia from hamsters, rabbits, dogs and large domestic species were not able to restore spermatogenesis fully in the immuno-deficient mouse testes [47-49]. The hamster to mouse transfer resulted in the production of abnormal hamster spermatozoa. Rabbit or dog spermatogonia repopulated the basal compartment of the host seminiferous tubules but were unable to undergo differentiation.

Spermatogonial transplantation offers interesting strategies for research on germ cells. It has become an assay to estimate the potential of germ cell development and a tool to determine whether the somatic environment or the germ cells are responsible for disturbed fertility of some transgenic animals (Fig. 1). For example, the importance of c-kit expression of spermatogonia and stem cell factor expression of sertoli cells was shown through transplantation experiments [50]. Transplanted estrogen receptor α (ERα) deficient germ cells into wild type testes, showed that the mutated germ cells induced qualitatively normal spermatogenesis. In contrast, after transplantation of germ cells from mice carrying the Juvenile spermatogonia depletion (jsd) mutation, no donor derived spermatogenesis can be established [51]. These studies opened new pathways for the detailed study of stem cell colonization and germ cell development.

6. A step forward: Production of transgenic animals

The proliferation of SSCs is rapid. Theoretically, one rat SSC can produce 4096 sperms and 1000 sperms can be produced per heart beat in humans [52]. If exogenous DNA is integrated into the chromosomes of SSCs, the sperm differentiated from these SSCs are likely to carry the foreign gene. When the egg becomes fertilized by the sperm with a foreign gene, there is a chance that offspring may carry the foreign gene. This foreign gene may then be duplicated with the proliferation of SSCs and retain for long time in stem cells. The differentiation and proliferation features of SSCs makes the generation of transgenic animals from these cells advantageous.

Different approaches with variable success have been tried for SSC-based production of transgenic animals [53]. As SSCs are located close to the basement membrane of the seminiferous tubules, the exogenous genes can be integrated into the chromosomes by seminiferous tubule microinjection. When testicular SSCs of mice and swine were transfected, lac-Z gene expression was detected in 8.0-14.8% of the infected seminiferous tubules with 7-13% sperms confirmed by PCR to carry the exogenous DNA [54] used electroportation as a method of introducing foreign genes with considerable success. The SSCs of seminiferous tubules in immature mice were transfected by retrovirus, and female mice produced transgenic offspring (2.8%) with foreign gene stably expressed [25].

The SSC transplantation provides an excellent approach to deliver exogenous gene carrying SSCs into the embryonic genome, and thus has a significant impact on the production of transgenic animals. When the primary culture of SSCs is carried out *in vitro*, transfection with foreign genes can be performed and subsequently the positive SSCs that carry the foreign genes can be identified through screening [55]. Rat SSCs were transduced *in vitro* with a lentiviral vector that expressed enhanced green fluorescent protein (EGFP), and then transplanted into the testes of immuno-deficient mice. The transduced rat SSCs produced EGFP-expressing spermatogenic cells, and micro-insemination using these cells was used to produce transgenic rats, which stably transmitted the transgene to the next generation [10]. Thus, xenogenic transplantation is a powerful strategy for transgenesis and smaller xenogenic surrogates can be used for male germ line modification using SSCs. A retroviral vector, carrying the EGFP reporter gene was applied to the short-term culture of dispersed testicular cells. After transplantation into sterilized recipient cockerels, these retrovirus-infected testicular cells restored exogenous spermatogenesis within 9 weeks with approximately the same efficiency as non-infected cells. Transduction of the reporter gene encoding EGFP was detected in the sperms of recipient cockerels with restored spermatogenesis [56]. This demonstrates that the transplantation of retrovirus-infected spermatogonia provides an efficient system to introduce genes into the chicken male germ line. When an adeno-associated virus carrying the GFP reporter gene was transfected into goat SSCs that were subsequently transplanted into recipient goat testes pre-treated with radiation, the transfected SSCs colonized the recipient testes and produced transgenic goat sperm. After fertilization *in vitro*, it was found that 10% of the embryos were transgenic, showing germ line transmission [57].

7. Prospects and Limitations

SSCs have attracted more attention due to their unique properties. In domestic animals, SSCs may facilitate the generation of transgenic farm animals and thus the ability to generate and preserve valuable animals such as rare breeding stock. SSCs can be used to enhance the economic value of livestock by improving the breeding efficiency, thereby resulting in better genetic merits, if the SSCs of livestock with high economic value are transplanted into receptor animals with relatively low economic value. SSC transplantation may also make breeding of elite male livestock in harsher environments possible as a result valuable offspring can be produced. To date, among non-rodent species, live progeny has been produced following SSCs in only goats and sheep, with an efficiency of about 7-10% [58, 36]. The field application

of this new technology for farm animals relies upon the efficient production of donor-derived offspring. Therefore, the feasibility and efficiency of live offspring production via SSCs in farm animals need to be investigated further. As compared to traditional microinjection technique, the embryonic stem cell method and sperm vector, SSCs-based production of transgenic animals is much simpler and also eliminates the use of specialized equipment and skilled operators. As SSCs can differentiate into many sperm cells, more transgenic offspring can be obtained through biomagnifications.

In addition to the rich prospects of SSC-based technology, it has several limitations. For example, although injection of seminiferous tubules is simple, the transfection efficiency of SSCs is relatively low in testes, resulting in lower rate of transgenic offspring. The transplantation efficiency of SSCs is high, but currently few species can achieve transgenic animals by this method, which relies on many SSC technologies such as isolation, purification, *in vitro* culture and preservation.

8. Conclusion

In conclusion, with further research on SSCs, these problems will eventually be resolved and the establishment of SSC-based methods will have a significant impact in terms of reproduction and production of traditional livestock and conservation of endangered species. Further these techniques will pave the way and ray of hope for individuals having the problems related to fertility especially oncological/cancer patients and options for fertility preservation in males.

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