OPINION

# Spermatogonial stem-cell transplantation: an alternative approach for transgenesis in farm animals

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*Genetic modification of spermatogonial stem cells followed by their transplantation to homologous recipients has shown immense potential to complement the current methods being used for generating transgenic farm animals. Using this technique, the time duration required to generate transgenic founder animals can be reduced at least by half, which would significantly reduce the generation interval. However, significant efforts need to be made to improve the efficiency of transgenic offspring production for its successful implementation in large-scale production of therapeutic proteins for human use.*

Production of transgenic farm animals has always fascinated the scientific community due to their potential use as models for biomedical research, organ and tissue donors for xenotransplantation in humans, and bioreactors for cost-effective production of pharmaceutical/therapeutic proteins useful for human health. To date, various approaches have been used to generate transgenic farm animals. Among them, pronuclear microinjection and somatic cell nuclear transfer (SCNT) are the most prominent methods currently being used. However, due to their low efficiency and high risk of mosaicism (pronuclear microinjection) and developmental abnormalities (SCNT) in the resultant offspring, transgenic technology could not be efficiently implemented in farm animals[1]. Therefore, the exploration of alternative strategies to achieve higher efficiency, and less time and capital requirement is of utmost priority.

In this context, genetic manipulation of spermatogonial stem cells (SSCs), the unipotent adult stem cells of the testis which form the base of male fertility, has demonstrated the potential to complement the current methods for transgenesis. *In vitro* genetic modification of SSCs and their subsequent transplantation to suitable homologous recipients has culminated in the production of donor-derived transgenic spermatozoa. The spermatozoa thus produced can be used to generate founder transgenic animals by artificial insemination (AI) or *in vitro* fertilization. In addition to transgenic research, SSC transplantation could play a crucial role to overcome infertility/subfertility in male farm animals.

Although initially developed for rodents, the successful generation of transgenic mice and rats has encouraged the SSC transplantation-based genetic modification in farm animals as well. Since SSCs are a rare population in the testis (~0.03% in adult mouse testis) and unique marker(s) for SSCs have not been identified yet, the success of SSC-based genetic manipulation relies upon their efficient isolation and enrichment. Currently, the isolation of SSCs is achieved by a two-step enzymatic digestion method using trypsin, collagenase, hyaluronidase and DNase I. However, further purification of SSCs is constrained by non-availability of a unique SSC marker. The only option is to enrich the SSCs based on their physical properties using differential plating and Percoll density gradient centrifugation. In recent advancements, fluorescence-activated cell sorting and magnetic-activated cell separation have also been successfully employed for the SSC enrichment using markers for undifferentiated type-A spermatogonia.

The next challenge is to ensure adequate supply of SSCs for their downstream application in genetic manipulation and transplantation. Therefore, the development of an efficient culture system for their *in vitro* propagation is deemed essential. Although researchers have succeeded to establish the long-term SSC culture in rodents, the same results could not be replicated in SSCs from farm animals due to limited knowledge of culture conditions and growth factor requirements for these species. To date, only short-term cultures for a duration of up to two months have been reported across farm animal species. Recently, we have optimized the growth-factor defined serum-free culture conditions for the *in vitro* propagation of buffalo SSCs[2].

For genetic modification of SSCs in culture, recombinant viral vectors such as lenti- and adeno-associated viral vectors are commonly used. Although these viral vectors offer high transduction efficiency for slowly dividing SSCs, the biosafety-related risks associated with their use are the major hurdles in applying the technique in the production of therapeutic proteins for human health. Therefore, it is recommended to explore suitable non-viral transfection methods offering high transfection efficiencies for SSCs. In this regard, we have recently reported ~68.55% transfection efficiency for the enriched buffalo SSCs using nucleofection, an improved version of electroporation[3]. The transfection efficiency of nucleofection was similar to that achieved using recombinant viral vectors, thus indicating its potential as a game-changer for genetic modification of SSCs during culture.

The age of SSC donors and recipients plays a significant role in determining the success of SSC transplantation. Special emphasis has been given to use prepubertal donors (3–6 months of age) because their testes naturally harbour a higher population of SSCs due to the absence of fully-fledged spermatogenesis. Concomitantly, the seminiferous tubules of immature recipients provide a better microenvironment for engraftment and proliferation of donor SSCs. Moreover, the absence of several layers of differentiating germ cells permits easy access of transplanted SSCs to the stem-cell niches present at the basolateral compartment, which further improves the transplantation efficiency. It has been reported that the depletion of endogenous spermatogenesis by busulfan treatment or X-ray irradiation improves the colonization efficiency in adults.

For SSC transplantation, the recipients are given epidural anaesthesia and restrained in lateral recumbency. Then the transplantation is performed under

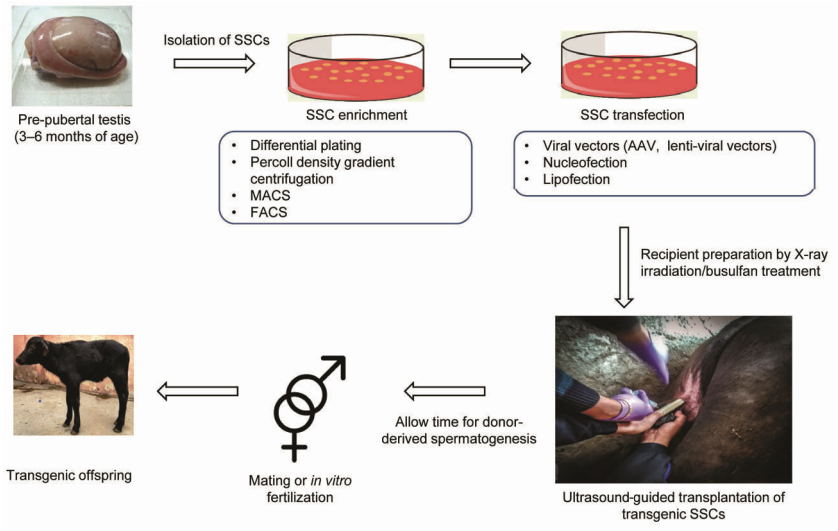


**Figure 1.** Schematic representation of steps involved in the generation of transgenic buffaloes by spermatogonial stem cell transplantation.

ultrasound guidance by visualizing the rete testis using an ultrasound probe and the donor SSCs are injected into the rete testis using a 22G needle (Figure 1). So far, live offspring following SSC transplantation have been produced in goats and sheep with approximately 7–10% efficiency[4]. Donor SSCs-derived spermatogenesis has been reported in pigs[5], cattle[6] and camel[7] as well. The transmission of transgene following the transplantation of transgenic SSCs has been successful in goats[8] and pigs[5]. Recently, we have optimized the homologous SSC transplantation in water buffalo using transfected enriched SSCs[3]. The donor cells were able to colonize in the recipient seminiferous tubules, proliferate and persisted till 7–8 weeks of transplantation, after which the recipient testes were collected for further analysis.

In conclusion, since its inception, SSC transplantation has shown tremendous potential and encouraging preliminary results for fertility restoration and transgenesis in farm animals. The time duration from SSC transplantation to the first detection of transgenic spermatozoa in the recipient's ejaculate is only a few months. Therefore, the duration for the generation of founder transgenic animals can be reduced at least by half compared to the current methods, which would significantly reduce the maintenance cost. However, the successful implementation of this technique at the field level would be determined by the efficient production of donor transgenic SSCs-derived offspring. For this, researchers have to further refine the efficiency and efficacy of this technique by focusing on the following crucial factors that affect the success of SSC transplantation in farm animals:

- Unique SSC markers need to be identified to obtain a highly pure population of SSCs in the donor cell population.
- Establishment of long-term SSC culture for farm animal species by optimizing the culture conditions and growth factor requirements.
- Improving the efficiency of non-viral transfection methods for genetic modification in SSCs to generate transgenic animals producing therapeutic proteins for human consumption.

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