Review Article

Recent Advances in Application of Male Germ Cell Transplantation in Farm Animals

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Transplantation of isolated germ cells from a fertile donor male into the seminiferous tubules of infertile recipients can result in donor-derived sperm production. Therefore, this system represents a major development in the study of spermatogenesis and a unique functional assay to determine the developmental potential and relative abundance of spermatogonial stem cells in a given population of testis cells. The application of this method in farm animals has been the subject of an increasing number of studies, mostly because of its potential as an alternative strategy in producing transgenic livestock with higher efficiency and less time and capital requirement than the current methods. This paper highlights the salient recent research on germ cell transplantation in farm animals. The emphasis is placed on the current status of the technique and examination of ways to increase its efficiency through improved preparation of the recipient animals as well as isolation, purification, preservation, and transgenesis of the donor germ cells.

1. Development of Male Germ Cell Transplantation Technique in Rodents

Germ cell transplantation (GCT), also referred to as spermatogonial stem cell (SSC) transplantation, is a powerful technology first introduced in 1994 by Brinster and colleagues. Although initially developed using a mouse model, GCT has important applications in the study and manipulation of spermatogenesis in many species. In this method, testis cells obtained from a fertile donor male are transferred into the seminiferous tubules of infertile recipient testes where donor-derived sperm production can occur, allowing the recipient to sire progeny [1, 2] (Figure 1). In essence, donor SSCs deposited in the lumen of the recipient seminiferous tubules are allowed by the Sertoli cells to migrate to the basolateral compartment of the tubule, to proliferate, form new colonies and initiate donor-derived spermatogenesis [3, 4]. Following the original introduction of GCT in mice [1, 2], the technique was also successful among rats [5, 6]. In laboratory rodents, GCT not only provides a unique opportunity for gaining new insight into spermatogenesis and the biology of the stem cell niche, but also presents a unique functional bioassay to test the competence of putative SSCs. Furthermore, GCT also offers a new strategy for preservation of male fertility and an alternative approach for generation of transgenic animals [7, 8].

2. Cross-Species Application of Male Germ Cell Transplantation

Rather surprisingly, cross-species (xenogeneic/heterologous) transplantation of testis cells from donor rats into recipient mice resulted in complete rat spermatogenesis [9]. This development sparked an interest in the idea of using the laboratory mouse as a universal recipient model for testis cells from different donor species. However, while hamster testis cells transferred into recipient mouse testes also developed complete donor-derived spermatogenesis [10], GCT from genetically more distant donor species, including farm animals, into mice only resulted in colonization or

proliferation of SSCs but not in complete spermatogenesis [11–15] (Table 1). This block in differentiation of donor germ cells is believed to be due to the incompatibility of donor germ cells and mouse Sertoli cells [16]. Although GCT from nonrodent species into the mouse testis did not result in complete spermatogenesis, it still is the only available bioassay for detecting the colonization potential of SSCs in a given population of donor testis cells from any species [11, 13, 17]. Interestingly, when (rather than transferring isolated testis cells into the seminiferous tubules) small fragments of testis tissue were transplanted under the back skin of recipient mice, complete donor-derived spermatogenesis was observed from a wide range of donor species, including farm animal species [18–26].

3. Cell Transfer Techniques

The original procedures described for the transfer of donor male germ cells into the seminiferous tubules of recipient mice could not be directly applied in farm animals. Therefore, we used ultrasound-guided cannulation of the centrally located rete testis [41] to infuse donor germ cells by gravity flow which was successful *in vivo* for pigs and goats [32, 33]. The procedure could be completed in 15–30 min and resulted in filling of about half of the recipient seminiferous tubules with donor cells. This methodology has been successfully adapted for use in rams and bulls *in vivo* [36, 40, 42]. Injection into the extratesticular rete testis using ultrasound guidance or surgical dissection was also reported to be applicable for use in rams [39, 43]. The success of the rete testis injection approach was further shown through donor-derived sperm production by the recipients [34, 44] and birth of progeny carrying the donor characteristics [34, 40].

4. Preparation of Recipient Animals

The success of GCT between unrelated laboratory rodents appears to depend on the availability of recipients of strain that are genetically compatible with the donor animals, are inherently immunodeficient, or have undergone immunosuppressive treatments [1, 45, 46]. Surprisingly, however, recipient pigs, goats, sheep, and bulls with fully functional immune systems did not reject germ cells from unrelated donors, making the practical application of the approach more feasible in farm animals [32–34, 39, 40].

Studies show that donor-derived spermatogenesis could be significantly improved when the recipient's endogenous germ cells are suppressed or depleted [6, 47]. Unlike in laboratory rodents, mutant animals with genetically impaired spermatogenesis are not readily available for use in work with farm animals. The azoospermic Klinefelter bull used in a study was determined not to be a useful recipient model for GCT [42], while limited donor-derived spermatogenesis was observed after GCT into two boars affected by the immotile short-tail sperm defect [37].

An alternative to the use of recipient animal models with congenital germ cell deficiency is removal of endogenous germ cells by cytoablative methods to facilitate further access to, and the availability of, the stem cell niche. We and others have achieved partial ablation of endogenous germ cells using busulfan (a chemotherapy agent) treatment of postnatal pigs [37, 48] and also during the *in utero* development of piglets [48]. This latter approach is particularly useful for preparation of piglets because treating a pregnant sow will result in producing multiple potential recipients at higher efficiency, but without the harmful health effects observed after treating postnatal piglets [48]. Where the facilities are available, local irradiation of testes can also be very effective in reducing the number of endogenous germ cells in recipient goats, rams and bulls [36, 48, 49].

For practical reasons, most researchers have used immature recipients for GCT in farm animals, while recipients for GCT in laboratory rodents are typically adults. The use of immature recipient testis not only facilitates access of the transplanted donor germ cells to the tubular basolateral compartment (because it lacks the hindering multiple layers of germ cells), it also provides a more favorable environment than adult testes for engraftment and expansion of donor germ cells [50]. In our previous studies, we used immature pig and goat recipients with no pretreatment [32–34], resulting in ~7% donor-derived progeny in goats [34], while after preparation of immature recipients with testis irradiation, the progeny rate averaged ~8% or 10% in sheep and goats, respectively, [27, 40]. These results may indicate that while gradual progress in the efficiency of GCT in farm animals is being achieved, recipient preparation of immature recipients may not be as critical as that for adult recipients [6, 50]. It may also be concluded that recipient preparation is only part of the requirements for a successful GCT, and that other aspects of the system also need to be optimized before a higher efficiency is expected.

5. Preparation of the Donor Germ Cells

The efficiency of GCT is also highly dependent on the relative abundance of SSCs transplanted [3, 51–53]. In the adult testis, SSCs are a rare population, making up only ~0.02% to 0.2% of testis cells in mice and rats [54–56]. The efficiency of colony establishment is also low, and only 1% [53] or 7–20% of the transplanted SSCs will actually colonize the recipient testis [3, 52, 53]. Naturally, increasing the number of SSCs in the donor population of cells prior to GCT will have a direct effect on the number of expected spermatogenic colonies in recipients [51, 52, 57].

5.1. Choice of Donors. For GCT in rodents, several strategies including the use of cryptorchid, vitamin-A deficient or Steel (Sl) mutant mice can be used to increase the proportion of nondifferentiated spermatogonia in the population of donor cells [58–61]. Surgical induction of cryptorchidism in the donor, 2-3 months prior to use in GCT, is a common strategy resulting in elimination of a large number of differentiated germ cells, and thereby up to 23-fold increase in the relative number of SSCs [51]. These strategies have not been well pursued for use in farm animal GCT, partly

TABLE 1: Summary of germ cell transplantation in different donor and recipient species.

| Donor	Recipient	Colonization of SSCs	Donor-derived			
			Spermatocytes	Spermatozoa	Offspring	Transgenic progeny
Mouse	Mouse	+	+	+ [2]	+	+ [1, 27, 28]
Rat	Rat	+	+	+	+	+ [29, 30]
Rat	Mouse	+	+	+ [9, 31]	—	—
Hamster	Mouse	+	+	+ [10]	—	—
Rabbit	Mouse	+	[11]	—	—	—
Dog	Mouse	+	[11]	—	—	—
Pig	Mouse	+	[13]	—	—	—
Cattle	Mouse	+	[13]	—	—	—
Horse	Mouse	+	[13]	—	—	—
Baboon	Mouse	+	[14]	—	—	—
Mouse	Pig	− [32]	−	—	—	—
Goat	Goat	+ [33]	+	+	+	+ [34]
Cattle	Cattle	+ [35]	+	+ [36]	—	—
Cat	Mouse	+	[12]	—	—	—
Pig	Pig	+ [32]	+	+ [37]	—	—
Dog	Dog	+	+	+ [38]	—	—
Sheep	Sheep	+	+	+ [39, 40]	+ [40]	—

+: positive results; −: negative results.
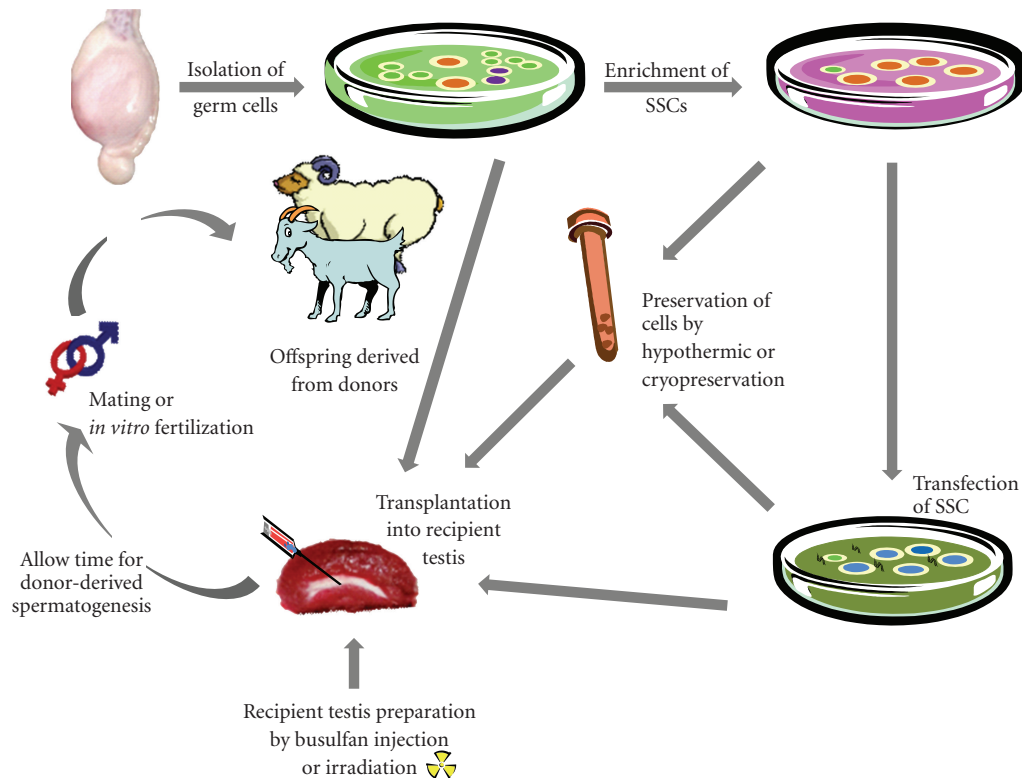
FIGURE 1: A schematic representation of procedures involved in germ cell transplantation in farm animals. A single-cell suspension of germ cells is prepared after enzymatic digestion of the donor testis for transplantation into recipient testes. Alternatively, the number of spermatogonial stem cells (SSCs) can be enriched in the donor cells and the resultant cells can be used fresh or preserved (for short term through hypothermic preservation or long-term by cryopreservation) and/or transfected with genes of interest before transplantation. The recipient animal can be treated with busulfan or undergo local irradiation of the testes to reduce the number of endogenous SSCs, in preparation for germ cell transplantation. Transplanted SSCs can form colonies of donor-derived spermatogenesis and produce sperm to allow the recipient to sire progeny carrying the donor haplotype.

to avoid the added complication of the procedure. On the other hand, testes from immature donors have been a frequent source of cells for use in farm animal GCT, because they provide a population of cells with a naturally higher proportion of germline stem cells than those of adults. In the neonatal testis, gonocytes are the only type of germ cells present, comprising 1-2% of total number of isolated testis cells in rodents [62, 63] or ~7% of intratubular cells in piglets [48]. Gonocytes have germline stem cell potential and upon transplantation into recipient testes are able to establish complete spermatogenesis [62]. Therefore, the neonatal/immature testis provides a logical source for GCT, especially for farm animals.

5.2. Isolation of Donor Germ Cells. Procedures for isolation of testis cells vary among laboratories, depending on the target cell types and the donor species. Two-step enzymatic digestion has been widely applied to isolate both gonocytes and SSCs in many species. In the first digestion step, collagenase and hyaluronidase enzymes are usually used to largely remove testis interstitial cells. As a second step, trypsin-EDTA (with or without additional enzymes) is used to break down seminiferous cords/tubules, while DNase is added to prevent cellular aggregation [64]. Using two-step digestion usually results in a maximum of 10% gonocytes/SSCs in the freshly isolated testis cells [62, 63, 65–70]. As outlined above, the proportion of SSCs can be increased using physically or genetically modified donors [58–61].

After a systematic evaluation of several potential factors affecting germ cell isolation, we recently developed a novel three-step strategy (combining vortexing and digestion) to isolate porcine testis cells with a gonocyte proportion of ~40%, (among fresh cells and before applying enrichment methods) [71] as compared with the conventional two-step enzymatic digestion resulting in collection of ~7% gonocytes.

5.3. Enrichment/Purification of Donor Germ Cells. Depending on the donor cells, diverse strategies can be used to enrich (but rarely purify) SSCs [72]. These strategies are generally based on fluorophore-labeled antibody separation of testis cells using fluorescent activated cell sorting (FACS) [51, 65, 68, 73–75], magnetic activated cell separation (MACS) [57, 65, 75–78], forward/side scatter measurements in FACS [51, 68, 79], density gradient centrifugation [43, 65, 67, 74, 80, 81], or differential plating [43, 65, 67, 74, 81]. Using these approaches, SSCs have been enriched to as high as 75% in the population of donor testis cells from farm animals [43, 65, 67, 74]. However, work on enrichment of gonocytes, as opposed to SSCs, has been limited to a few reports in rodents and pigs [69, 82–84]. We recently assessed the efficiency of porcine gonocyte enrichment using density gradient and differential plating strategies. We found that gonocytes can be enriched to more than 80% using either Nycodenz density gradient or differential plating (with fibronectin and poly-D-lysine coatings), and to a purity of more than 90% by combining the two strategies (Yang and Honaramooz, unpublished data).

5.4. Preservation of Donor Testis Tissue and Cells. Different from the situation for GCT in rodents, preparation of the required high numbers of germ cells from testes of donor farm animals for same day transplantation could be a time-management challenge. Aside from the time needed for collection of donor testes from farm animals, the high volume of the tissue and presence of dense connective tissue make the process of digestion time consuming and often requiring the tissue or cells to be stored overnight before GCT [32, 39, 40, 43, 74]. 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, not only is it not suitable for short-term preservation, it can also damage cells, as cryopreservation of bovine germ cells resulted in cell survival rates of ~50–70% [85]. We also showed the feasibility of cryopreserving fragments of porcine testis tissue [86, 87] resulting in a postdigestion cell survival of ~55–88% while maintaining the *in vivo* developmental potential [86].

Short-term preservation of testis cells and tissue, on the other hand, is necessary and could be more suitable for immediate applications such as in GCT and for shipment of cells/tissue between collaborating laboratories. Hypothermic temperatures (above freezing point but below the body temperature) cause a decrease in cellular metabolism rate, oxygen demand, and energy consumption and therefore prolong cell viability. Ice-cold storage of testis tissue for 1 or 2 days was suggested to improve donor-derived spermatogenesis after xenografting [87, 88]. Testis tissue stored at refrigeration temperature for 3 days maintained its structural integrity [89], but cell viability starts to decrease [87]. We recently showed that using proper media, isolated cells from donor piglets could be maintained even at room temperature for at least 24 hours and up to 6 days at refrigeration temperature, resulting in up to 88% after-storage cell viability, and without changing the germ cell proportions or cell culture properties [89, 90].

5.5. Identification of Donor Cells after Transplantation. The availability of donor models providing cells with visual markers was critical for original development of GCT technique in mice [1, 2], because they allowed monitoring of the long-term fate of donor cells. Similar donor models are not readily available for use in farm animal GCT. Therefore, to track donor cells, researchers largely rely on fluorescence labeling of donors cells (although only traceable for a short period of time) [32, 39, 43] or use genetic identification of donor-derived cells/sperm. As a proof-of-principle study, we transplanted testis cells from transgenic donor goats into testes of wild-type recipient goats, resulting in production of donor-derived sperm and subsequently transgenic progeny [34]. Microsatellite detection has also been used to identify donor-derived sperm DNA in the semen of recipient rams [44]. Alternatively, transplantation of normal testis cells into recipients with genetic aberrations such as the immotile short-tail sperm syndrome has been

used to facilitate detection of normal pig sperm in the semen of recipient pigs as indication of GCT success [37].

5.6. Insertion of Genes into Donor Germ Cells. All adult stem cells have the ability to self-renew and to produce differentiated cells; however, SSCs are unique because they produce a lifetime supply of sperm in adults with the potential to contribute genes to progeny. Therefore, if SSCs are incorporated with genes of interest prior to transplantation, their resultant sperm will carry the transgene in the recipient. The desired genes for example can include those of visual markers to facilitate GCT studies and to serve as a step toward generating transgenic farm animals through GCT. Methods of DNA delivery into cells include chemical, lipofection, electroporation, and viral vectors. Compared with nonviral methods, recombinant viral vectors generally have very high cell transfection efficiency rates, and some are able to integrate the transgene into the SSC genome for stable expression. However, construction of the desired viral vectors is more involved and their use requires a facility with a higher biosafety level. Nonviral alternatives, on the other hand, are relatively easy to use and pertain virtually no biosafety risk to the operator or the public but their efficiency rates are usually much lower.

The work in transgenesis of germ cells for farm animal GCT is at early stages. Electroporation of bovine testis tissue *in vitro* resulted in transfection of SSCs shown after xenografting into the back of immunodeficient mice [91]. Transfection of pig germ cells was also reported after injection of plasmids mixed with a lipofection reagent into busulfan treated testes; however, it was unclear from the report whether SSCs were indeed transfected [92].

In a preliminary study, we used a recombinant adenoassociated virus (rAAV) containing the GFP reporter gene and CMV promoter to transfect isolated pig testis cells *in vitro* and observed GFP expression in cultured cells and in spermatogenic colonies for several months after GCT into recipient testes [93]. Subsequent use of rAAV vectors carrying the GFP for transfection of goat testis cells prior to GCT into recipient goats led to long-term expression of GFP in recipient testes and presence of transgenic sperm in 35% of the ejaculates. When semen from these recipient goats was used for *in vitro* fertilization, 10% of embryos were transgenic, showing germline transmission [27]. These results indicate that transgenesis via GCT in farm animals is a promising approach to generate transgenic animals.

6. Potential Applications of Male Germ Cell Transplantation in Farm Animals

Although technically still at an experimental level, there are significant potential applications for GCT in farm animals. This includes genetic modification of farm animals through transplantation of genetically altered male germ cells for improving productivity traits or producing transgenic farm animals. As such, GCT would be an alternative strategy to the currently inefficient and costly methods of generating transgenic farm animals [94, 95]. The development of

transgenic pigs, for instance, is of interest because of its potential to provide tissues and organs for xenotransplantation to humans and as a model for biomedical research [96]. Genetically altered dairy goats, sheep, and cows are also of significant economic value for the production of biopharmaceutical proteins in their milk [95, 96]. It has also been proposed that GCT can be used as an alternative way to artificial insemination for dissemination of elite sire bull genetics in extensive beef cattle grazing systems where animal handling is a limiting factor [35, 97]. Another important potential application of GCT is to restore fertility by cryopreservation followed by transplantation of genetic material from immature males (as shown for testis tissue grafting [86]) of rare or valuable livestock breeds that die while they are premature or from horses that undergo early castration but display superior traits later in life. The advantage of using SSCs for genetic dissemination or fertility restoration is that SSCs can be harvested, cultured, propagated, cryopreserved, or transfected and still preserve the potential to colonize the recipient testes [1, 27, 28, 32, 34, 98–102]. Since SSCs can both self-renew and produce a lifetime supply of sperm in adults, they have tremendous potential in modifying the male germ line as compared with, for example, the use of a finite amount of frozen semen from a given donor.

7. Production of Live Progeny after Male Germ Cell Transplantation

The birth of offspring carrying the donor haplotype after mating of a recipient can be viewed as the most convincing evidence for any successful GCT. In the initial report of GCT in mice, some of the infertile recipients produced enough donor-derived sperm to allow them to sire progeny [1]. As summarized in Table 1, among nonrodent species, live progeny has so far been produced following GCT in only goats and sheep, with an efficiency of ∼7–10% [34, 40]. The field application of this new technology for farm animals relies upon the efficient production of donor-derived offspring in the end. Therefore, the feasibility and efficiency of live offspring production via GCT in farm animals need to be investigated further and improved (Figure 1).

8. Conclusions and Future Directions

In a short period of time since the first reports in 2002-2003 [32–34, 36, 42], GCT in farm animals has shown great potential and promising initial results. Currently the percentage of donor-derived sperm in the semen of recipients after GCT is rather low, but evidence indicates that the efficiency can be improved. As we move beyond the stage of proof-of-principle studies, it is expected that the research in this area will focus more on improving a number of factors related to the success rate of the technique and on making it a viable option for downstream applications. These improvements are especially needed for obtaining higher numbers and purity of SSCs in the donor populations of cells. Another area of high potential is work on increasing the

efficiency of nonviral transfection of germ cells, particularly if the ultimate transgenic animals are to be considered for generating products for human consumption. There are several advantages in pursuing farm animal transgenesis through GCT. For instance, the time from GCT into prepubertal recipient farm animals to first detection of transgenic sperm in the ejaculate is only a few months. Therefore, the time required to start collecting transgenic sperm may be reduced by one generation to about one half of that required by current methods. This time saving factor alone can significantly reduce the maintenance cost and accelerate the generation of a transgenic herd needed for large-scale production.

Acknowledgments

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