

# Germ cell transplantation in pigs – advances and applications

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Transplantation of germ cells from fertile donor mice to the testes of infertile recipient mice results in donor-derived spermatogenesis and transmission of the donor haplotype to offspring of recipient animals. In the pig, germ cells can be transplanted to a recipient testis by ultrasound-guided cannulation of the rete testis with delivery of cells by gravity flow. It is important to note that germ cell transplantation was successful between unrelated, immuno-competent pigs, whereas transplantation in rodents requires syngeneic or immuno-compromised recipients. Efficiency of colonization of the recipient testis by donor-derived germ cells can be improved by pretreatment of the recipient animal to deplete endogenous germ cells. Genetic manipulation of isolated germ line stem cells and subsequent transplantation will result in production of transgenic sperm. Transgenesis through the male germ line has tremendous potential in species like pigs where embryonic stem cell technology is not available and current options to generate transgenic animals are inefficient. Introduction of a genetic change prior to fertilization will circumvent problems associated with manipulation of early embryos and developmental abnormalities associated with somatic cell nuclear transfer and reprogramming. Viral transduction of germ cells prior to transplantation has been used to generate transgenic rodents and has also shown early promising results in pigs. Current research is directed toward improving protocols for isolation and culture of porcine male germ cells to increase efficiency of transgene transmission and to allow for gene targeting prior to germ cell transplantation. It is expected that germ cell transplantation will then provide a viable alternate approach to generate germ line transgenic pigs.

## **Germ cell transplantation – basic aspects**

Spermatogenesis is a continuous, highly organized process comprised of sequential steps of cell proliferation and differentiation resulting in production of virtually unlimited numbers of spermatozoa throughout the life of the male (Russell *et al.*, 1990). The foundation of this system is the spermatogonial stem cell, which has the unique potential for both self-renewal and production of differentiated daughter cells, and will ultimately form spermatozoa (Huckins, 1971; Clermont, 1972; Meistrich and van Beek, 1993). Among stem cells in a male individual,

the spermatogonial stem cell is unique in that it is the only cell in an adult body that divides and contributes genes to subsequent generations making it a perfect target for genetic manipulations.

In 1994, Dr. Ralph Brinster and colleagues at the University of Pennsylvania reported that transplantation of germ cells from fertile donor mice to the testes of infertile recipient mice resulted in donor-derived spermatogenesis and sperm production by the recipient animal (Brinster and Zimmermann, 1994). Use of donor males carrying the bacterial  $\beta$ -galactosidase gene allowed for identification of donor-derived spermatogenesis in the recipient mouse testis and established the fact that the donor haplotype was passed on to offspring by recipient animals (Brinster and Avarbock, 1994).

In 1995, Jiang and Short applied the technique to germ cell transplantation between rats (subsequently also reported by Ogawa *et al.*, 1999a; and Zhang *et al.*, 2003), and in 1996, Brinster's group showed that mouse spermatogonial stem cells, cryopreserved for prolonged periods of time before transplantation, still established spermatogenesis in the recipient testis (Avarbock *et al.*, 1996).

In recent years, germ cell transplantation studies provided new insights into different aspects of spermatogenesis. Germ cell transplantation in rodents made it possible to study the stem cell niche in the testis, and to characterize putative spermatogonial stem cells (Parreira *et al.*, 1998; Nagano *et al.*, 1999; Ventela *et al.*, 2002). Cross-species transplantation established that the cell cycle during spermatogenesis is controlled by the germ cell and not the Sertoli cell (Franca *et al.*, 1998). Also, sperm arising from transplanted donor germ cells were capable of fertilization in vivo and in vitro (Brinster and Avarbock, 1994; Goossens *et al.*, 2003; Honaramooz *et al.*, 2003a). Recently, transplantation experiments even demonstrated the developmental potential of mouse primordial germ cells to initiate spermatogenesis when transplanted into a post-natal testis (Chuma *et al.*, 2005).

### Xenotransplantation of germ cells

In 1996, production of rat sperm in mouse testes was achieved following cross-species (xenogeneic) spermatogonial transplantation from rats to mice (Clouthier *et al.*, 1996) and was subsequently successful from mice to rats (Ogawa *et al.*, 1999a; Zhang *et al.*, 2003). For its obvious practical potential, cross-species germ cell transplantation was explored further. Hamster spermatogenesis occurred successfully in the mouse host (Ogawa *et al.*, 1999b); however, with increasing phylogenetic distance between donor and recipient species, complete spermatogenesis could no longer be achieved in the mouse testis. Transplantation of germ cells from non-rodent donors ranging from rabbits and dogs to pigs and bulls, and ultimately non-human primates and humans, resulted in colonization of the mouse testis, but spermatogenesis became arrested at the stage of spermatogonial expansion (Dobrinski *et al.*, 1999, 2000; Nagano *et al.*, 2001a, 2002a). It appears that the initial steps of germ cell recognition by the Sertoli cells, localization to the basement membrane, and initiation of spermatogonial proliferation are conserved between evolutionary divergent species. However, it was hypothesized that with increasing phylogenetic distance between donor and recipient species, the recipient testicular environment (comprised of Sertoli cells and paracrine factors) becomes unable to support spermatogenic differentiation and meiosis. This incompatibility of donor germ cells and recipient testicular environment was overcome by co-transplantation of germ cells and Sertoli cells (Shinohara *et al.*, 2003) or by testis tissue transplantation (Honaramooz *et al.*, 2002). Although xenogeneic spermatogonial transplantation did not have the envisioned immediate practical application, it nonetheless provides a bioassay for stem cell potential of germ cells isolated

from other species (Dobrinski *et al.*, 1999; 2000; Izadyar *et al.*, 2002) as well as an intriguing tool to study cellular and non-cellular requirements of spermatogenesis in diverse species.

### Germ cell transplantation in pigs

#### Technical aspects

The application of germ cell transplantation technology to non-rodent species was initially difficult. Due to differences in testicular anatomy and physiology, germ cells cannot be delivered by the same technique as in rodents. Instead, we developed a technique combining ultrasound-guided cannulation of the centrally located rete testis with delivery of germ cells by gravity flow. Fig. 1 outlines the different steps of the germ cell transplantation approach in the pig. With this technique, we succeeded in transplanting donor cells from pigs and goats to the testes of prepubertal recipient animals (Honaramooz *et al.*, 2002; 2003a, b). Transplantation of donor cells from transgenic goats provided proof-of-principle that germ cell transplantation results in donor-derived sperm production and fertility in a large animal species (Honaramooz *et al.*, 2003b). It is of interest to note that successful germ cell transplantation in rodents, and perhaps cattle, requires that recipient animals are either closely related to the donor or immunosuppressed (Kanatsu-Shinohara *et al.*, 2003a; Zhang *et al.*, 2003; Izadyar *et al.*, 2003), whereas germ cell transplantation in pigs and goats was also successful between unrelated individuals (Honaramooz *et al.*, 2002; 2003a, b). Experiments are now directed at improving the efficiency of germ cell transplantation in domestic animals by enrichment for stem cells and suppression of recipient spermatogenesis as discussed below.

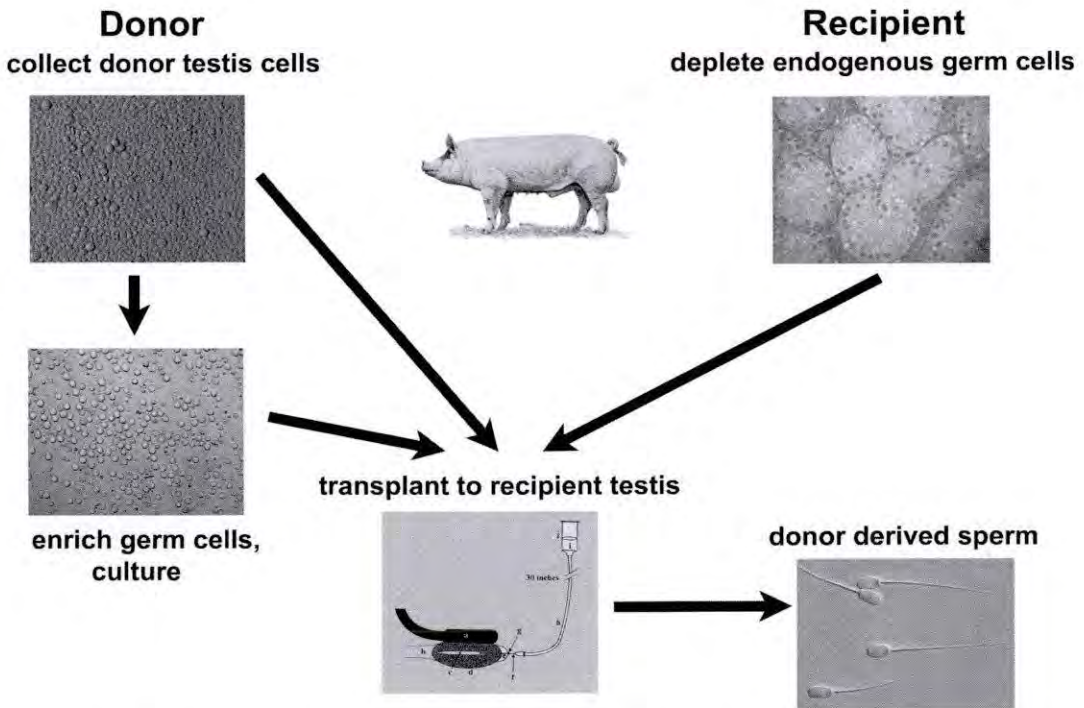


Fig. 1 Schematic representation of germ cell transplantation in the pig

### Recipient animal preparation

On the recipient side, using young mice and treating recipients with GnRH-agonists improved donor cell colonization (Shinohara *et al.*, 2001, Ogawa *et al.*, 1998, 1999a; Dobrinski *et al.*, 2001). Efficiency of colonization of seminiferous tubules by the transplanted germ cells can be optimized, if the recipient testes have little or no endogenous spermatogonia. Busulfan, a DNA alkylating agent that destroys proliferating cells, is frequently used in rodents to deplete recipient germ cells prior to germ cell transplantation. However, the sterilizing dose of busulfan is species- and strain-specific, and treatment can be lethal due to severe bone marrow depression (Ogawa *et al.*, 1999a; Brinster *et al.*, 2003). We, therefore, designed a study to develop a practical protocol for depletion of endogenous spermatogonia in potential recipient pigs. Treatment of pigs with busulfan between 5 and 17 weeks of age resulted in a significant reduction of testis weight and germ cell number, but also in systemic toxicity. Therefore, treatment *in utero* was explored as previously described in the mouse (Brinster *et al.*, 2003). Administration of busulfan to pregnant sows at days 98 and 108 of gestation (a period of high proliferation of foetal gonocytes) resulted in depletion of gonocytes with no observed adverse effects on the piglets or on sow health or fertility (Honaramooz *et al.*, 2004). The number of germ cells was significantly reduced at 2 – 8 weeks of age compared to untreated controls. At 16 weeks of age, when recipient pigs would be used for transplantation, only 8% of seminiferous tubules showed initiation of spermatogenesis compared to 100% in controls. It was shown previously in rodents that a complete suppression of spermatogenesis might not be desirable in recipient animals as some residual endogenous spermatogenesis is an indication that the testicular environment has not been permanently damaged by the cytotoxic treatment (Brinster *et al.*, 2003). Therefore, late gestation *in utero* treatment of pigs with busulfan provided a suitable testis environment for subsequent germ cell transplantation.

### Germ cell culture

It has been estimated that there are only about  $2 \times 10^4$  stem cells in  $10^8$  cells of a mouse testis (Meistrich and van Beek, 1993; Tegelenbosch and de Rooij, 1993). In order to study the biology of spermatogonial stem cells *in vitro* and to increase colonization efficiency after transplantation *in vivo*, it is desirable to obtain populations of cells enriched in spermatogonial stem cells from testis cell preparations. In the mouse, selection of germ cells for expression of  $\alpha_6$ - and  $\beta_1$ -integrin in the absence of c-kit receptor, as well as collection of cells from experimentally induced cryptorchid testes, resulted in a significant enrichment for spermatogonial stem cells (Shinohara *et al.*, 1999, 2000a, b). More recently, expression of either Thy-1, CD9 or Egr3, surface proteins, also expressed on embryonic or bone marrow stem cells, was utilized as markers for enrichment of mouse germ line stem cell populations (Kubota *et al.*, 2003, 2004; Kanatsu-Shinohara *et al.*, 2004; Hamra *et al.*, 2004). One previous report in pigs described the use of sedimentation at unit gravity (Staput) to obtain cell fractions highly enriched in type A spermatogonia (Dirami *et al.*, 1999). However, the approaches based on flow sorting, magnetic cell sorting or Staput to obtain the larger numbers of germ cells needed for transplantation are not very practical in the pig. Therefore, we are currently exploring enrichment strategies based on differential adhesion to plastic and extracellular matrix as previously reported in rodents (Shinohara *et al.*, 1999; Orwig *et al.*, 2002a). Early experiments showed promising results with porcine germ cells. In addition, donor age affects the number of germ cells present in the testis and also the relative number of putative spermatogonial stem cells in the total cell population (Shinohara *et al.*, 2001). Harvesting donor germ cells from animals just before puberty when spermatogonia and Sertoli cells are the only cell types present in the seminiferous tubules will assure that the relative number of germ cells collected from the donor testis will be maximized.

Nagano *et al.* (1998) were the first to show that mouse germ line stem cells could be maintained in culture for a long period of time. Improving culture conditions for male germ line stem cells is still under intense study as evidenced by recent reports of improved culture systems for mouse germ cells (Kanatsu-Shinohara *et al.*, 2003b, 2005; Jeong *et al.*, 2003). Co-culture with embryonic fibroblast or bone marrow stromal cells, but not Sertoli cell lines, and addition of several growth factors known to be beneficial for culture of other stem cell types or primordial germ cells, such as glial cell-line derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF), successfully maintained mouse germline stem cells in culture for varying periods of time (Nagano *et al.*, 1998; 2003; Kanatsu-Shinohara *et al.*, 2003b). Recently, Dr. Brinster's group reported an efficient long-term culture system for mouse spermatogonial stem cells (Kubota *et al.*, 2004) that is now being tested for its potential to maintain pig germ cells *in vitro*. Immortalized cell lines would provide tremendous potential for the study and manipulation male germ cells *in vitro*. To date, there are reports of three immortalized germ cell lines from rat and mouse (van Pelt *et al.*, 2002; Feng *et al.*, 2002; Hofmann *et al.* 2005), and similar immortalization strategies might hold promise for porcine germ cells.

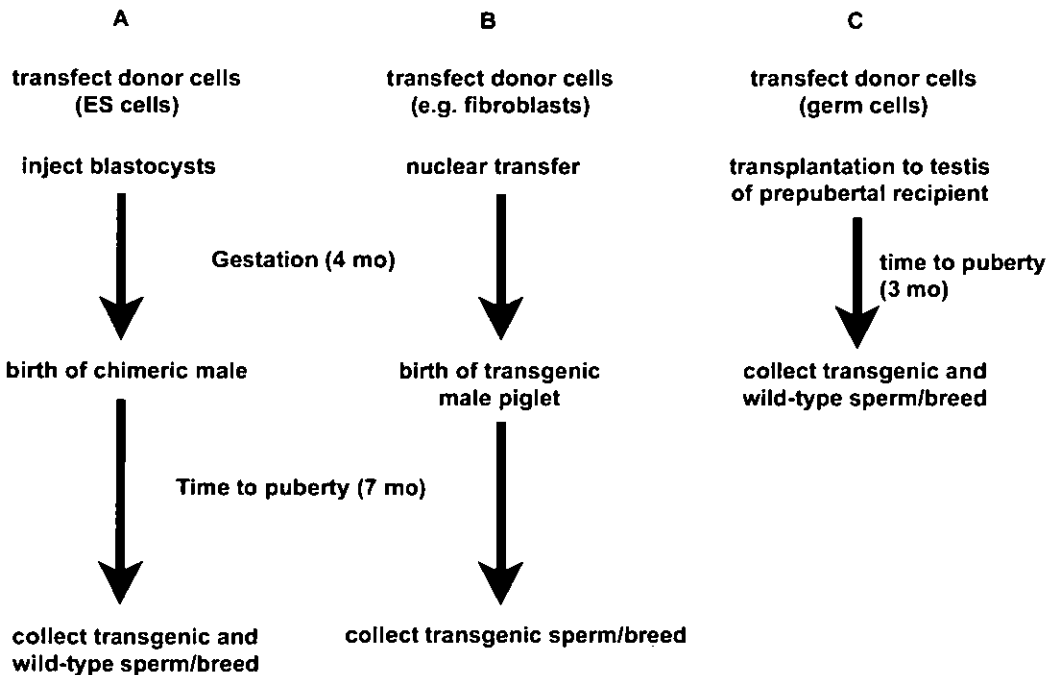
### Applications of germ cell transplantation in pigs

Germ cell transplantation is a powerful approach to study control of spermatogenesis with the ultimate goal to enhance or suppress male fertility. It also provides an opportunity to preserve genetic material of valuable males. Germ cell transplantation has an advantage over the only approach currently available, cryopreservation of sperm, in that it can be applied to prepubertal animals where sperm cannot be obtained or even to adults rendered azoospermic or teratozoospermic by disease. It has also been discussed as a tool to store genetic material from barrows used in performance testing in lieu of using littermates of the top quality animals for breeding. Another potential application would be delivery of genetic material to a closed production herd through resident recipient animals.

Perhaps the most intriguing application of the technique will be the transplantation of transfected germ cells as an alternate means to generate transgenic animals. Although to date the lack of pure starting populations of germ line stem cells and optimized culture systems have made this a difficult task, some success was reported in generating transgenic mice and rats by retroviral or lentiviral transduction of germ cells prior to transplantation (Nagano *et al.* 2001b, 2002b; Hamra *et al.*, 2002; Orwig *et al.*, 2002b). In one report, a transgene introduced using a retroviral vector was stably integrated and passed on to subsequent generations without gene silencing (Nagano *et al.*, 2001b). Since putative stem cells are considered to be replicating very slowly or not at all when cultured, viral gene therapy vectors that integrate into non-replicating cells are an obvious choice for gene transfer. Recombinant adeno-associated virus (rAAV) is a replication-defective, non-pathogenic human parvovirus that stably and site-specifically integrates into dividing and non-dividing human cells. It infects cells of different species, and therefore, does not require pseudotyping, readily integrates into quiescent cells in a short period of time and does not carry the same level of biosafety concerns as lentiviral vectors. Transduction of porcine germ cells using rAAV prior to transplantation showed promising results (Honaramooz *et al.*, 2003c), and is a topic of current investigation. However, AAV vectors accommodate only relatively small genetic insertions and have the potential for episomal integration and gene silencing. Therefore, use of other viral and non-viral transfection systems still needs to be further explored in porcine germ cells.

Practical application of transgenesis through germ cell transplantation is ultimately dependent on improvements in efficiency of stem cell selection, transfection and culture. Efficient

culture systems that support maintenance and expansion of stem cells are of utmost importance as they would permit gene targeting approaches and cell selection prior to transplantation. Transgenesis through the male germline using transplantation of genetically modified germ cells has tremendous potential in species like pigs where embryonic stem cell technology is not available. Current options to generate genetically modified pigs include pronuclear micro-injection of DNA (Hammer *et al.*, 1985) and nuclear transfer technology using modified donor cells (Lai *et al.*, 2002; Park *et al.*, 2002; Hyun *et al.*, 2003; Phelps *et al.*, 2003; Kolber-Simonds *et al.*, 2004), as well as sperm mediated DNA transfer (Lazzereschi *et al.*, 2000; Lavitrano *et al.*, 2002, 2003). However, currently available technology is frequently fraught with low efficiency and developmental abnormalities in the few resulting offspring, making the approach of using germ cell transplantation a potentially very valuable alternative. Introduction of a genetic modification prior to fertilization will circumvent problems associated with manipulation of gametes and early embryos and developmental abnormalities associated with nuclear reprogramming. In addition, even if embryonic stem cell technology becomes available for pigs, the time required until transgenic sperm can be harvested will be significantly shorter using germ cell transplantation (Fig. 2).



**Fig. 2** Comparison of three different approaches to transgenesis. A: Transgene introduction through embryonic stem cells (Note: This has been adapted in principle from work in rodents as the technology is currently not available in pigs); B: Transgenesis through somatic cell nuclear transfer (cloning); C: Transgenesis through germ cell transplantation (work in progress).

Introduction of a genetic modification before meiosis will allow for recombination to occur. A recipient animal, therefore, can produce sperm with different transgene integrations that may permit screening of offspring to select the most desirable genotype. Finally, genetic manipulation of male germ line stem cells may provide an approach to generate males that produce unisexual sperm by introducing a sex-linked mutation that selectively prevents formation of hap-

loid cells carrying either an X or a Y chromosome. Production of all male or all female offspring is of economic interest to different production systems. In addition, in the pig it might provide an avenue to avoid routine castration of male piglets, a procedure that is coming under increasing scrutiny due to animal welfare concerns.

### Conclusions

Germ cell transplantation was initially developed in rodents. Application to a large domestic animal species was first reported in the pig and subsequently in goats and cattle. Important aspects of the approach, including isolation of donor cells, delivery to recipient testes and recipient animal preparation have been established in the pig while others such as long-term culture and expansion of germ cells in vitro and efficient introduction of stable genetic changes are still under investigation. Germ cell transplantation allows for preservation of male genetic material and introduction of genetic changes through the male germ line. It represents an approach to germ line manipulation through use of transgenic sperm for natural breeding that is potentially more efficient than currently existing strategies.

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