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Transplantation of germ cells and testis tissue to study mammalian spermatogenesis[1]

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Abstract

It was first reported in 1994, that transplantation of germ cells from fertile donor mice to the testes of infertile recipient mice can result in donor-derived spermatogenesis and transmission of the donor haplotype to offspring of recipient animals. More recently, germ cell transplantation was successfully performed also in large animals. Importantly, germ cell transplantation was successful between unrelated, immuno-competent large animals, whereas efficient donor-derived spermatogenesis 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 domestic animal species 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. It is expected that germ cell transplantation will provide a viable alternate approach to generate germ line transgenic domestic animals. As an alternative to transplantation of isolated germ cells to a recipient testis, ectopic grafting of testis tissue from diverse mammalian donor species, including primates, into a mouse host represents a model to study spermatogenesis, to investigate the effects of substances with the potential to affect male fertility, and to produce fertile sperm from immature donors. Therefore, transplantation of germ cells or testis tissue are uniquely valuable approaches for the study, preservation and manipulation of male fertility in mammalian species.

Keywords: testis, germ cells, transplantation, grafting.

Introduction

Spermatogenesis is a continuous, complex process of cell proliferation and differentiation resulting in production of virtually unlimited numbers of spermatozoa throughout the adult life of the male (Russell *et al*., 1990). The foundation of this system is the spermatogonial stem cell which has the potential for both self-renewal and production of differentiated daughter cells which 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 mitotically and contributes genes to subsequent generations making it a perfect target for genetic manipulations.

In 1994, Brinster and colleagues 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 β-galactosidase gene allowed for identification of donor-derived spermatogenesis in the recipient mouse testis and established the fact that the donor haplotype is passed on to offspring by recipient animals (Brinster and Avarbock, 1994). Sperm arising from transplanted donor germ cells are capable of fertilization in vivo and in vitro (Brinster and Avarbock, 1994; Goossens *et al*., 2003, 2006; Honaramooz *et al*., 2003b)

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 the years since its initial report, 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; Kubota *et al.*, 2003; Nagano, 2003; Hamra *et al.*, 2004). Cross-species transplantation established that cell cycle during spermatogenesis is controlled by the germ cell and not the Sertoli cell (França *et al.*, 1998). 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). When presented with a phenotype of male infertility with a defect in spermatogenesis, it is often difficult to determine

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whether the defect originates in the germ cells, or in the somatic components of the testis (Ogawa *et al.*, 2000). To characterize an unknown defect, standard experimental design now includes reciprocal transplantation of germ cells from affected donors to wild-type testes and vice versa. Using this approach, germ cell transplantation was successfully applied to characterize the role of the c-kit receptor and its ligand stem cell factor in regulation of germ cell proliferation (Ohta *et al.*, 2000), to show that the defect associated with the juvenile spermatogonial depletion (*jsd*) mutation was inherent to the germ cells (Boettger-Tong *et al.*, 2000; Ohta *et al.,* 2001), that germ cells do not require estrogen receptors (Mahato *et al.*, 2000) or androgen receptors (Johnston *et al*., 2001) for development, and that germ cell differentiation is regulated by glial cell-line derived neurotrophic factor (GDNF; Creemers *et al.*, 2002; Yomogida *et al.*, 2003), *plzf* (Buaas *et al.*, 2004; Costoya *et al.*, 2004)and CREM function (Wistuba *et al*., 2002). Transplantation of wild-type germ cells into the testes of *Dazl* null mice established that the somatic compartment of the *Dazl* null testes remains functional (Rilianawati *et al.*, 2003). In the rat, germ cell transplantation experiments elucidated the defect underlying the *as*-mutation (Noguchi *et al.*, 2002).

Germ cell culture

Nagano *et al.* (1998) showed first that stem cells could be maintained in culture for a long period of time. 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 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). Efficient long-term culture systems for mouse and rat spermatogonial stem cells have now been described (Kubota *et al.*, 2004; Kanatsu-Shinohara *et al.*, 2005; Hamra *et al*., 2005; Tenenhaus *et al*., 2006). Recently, the developmental plasticity of cultured male germ cells was highlighted by reports that pluripotent stem cells could be isolated from cultures of neonatal and adult mouse testis cells (Kanatsu-Shinohara *et al*., 2003c; 2004 ; Guan *et al*., 2006) and by the demonstration that adult male germ line stem cells can even give rise to fertilization competent eggs when transplanted into an undifferentiated gonad in fish (Okutsu *et al*., 2006).

The majority of the work to date was performed with primary cultures of putative male germ line stem cells. While progress in this area has been significant, availability of immortalized cell lines would provide tremendous potential for the study and manipulation male germ cells in vitro. To date, there are reports of immortalized germ cell lines from rat and mouse (van Pelt *et al*., 2002; Feng *et al.*, 2002; Hofmann *et al*., 2005).

Transplantation of germ cells from different mammalian species to mouse testes

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). Recently, it was confirmed that rat sperm produced in a host mouse testis are capable of supporting normal development when introduced into rat oocytes by ICSI (Shinohara *et al.*, 2006). Hamster spermatogenesis also occurred successfully in the mouse host (Ogawa *et al*., 1999b); however, with increasing phylogenetic distance between donor and recipient species, meiotic differentiation 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, the testicular environment (Sertoli cells and paracrine factors) of the recipient mouse appears to be unable to support spermatogenic differentiation and meiosis from donor species other than rodents. This incompatibility of donor germ cells and recipient testicular environment could theoretically be addressed by co-transplantation of germ cells and donor Sertoli cells to the mouse testis (Shinohara *et al.*, 2003), and complete spermatogenesis from different mammalian species in a mouse host was achieved by testis tissue transplantation (Honaramooz *et al.*, 2002b, see below). Although xenogeneic spermatogonial transplantation did not result in spermatogenesis from donor species other than rodents, 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).

Germ cell transplantation in non-rodent species

Application of germ cell transplantation technology to species other than rodents has so far been reported in pigs, goats, cattle, monkeys and recently fish and chickens (Honaramooz *et al*., 2002a, 2003a; b; Mikkola *et al.*, 2006; Izadyar *et al*., 2003; Schlatt *et al*., 2002b; Takeuchi *et al*., 2003; Yoshizaki *et al*., 2005; Okutsu *et al*., 2006; Lee *et al*., 2006). While direct

injection of donor cells into rodent seminiferous tubules is possible via the efferent ducts, this is not feasible in larger mammalian species. Instead, a combination of ultrasound-guided cannulation of the centrally located rete testis with delivery of germ cells by gravity flow (Honaramooz *et al*., 2002a, 2003b) was shown to be a successful approach in large animals. When germ cells from transgenic donor goats were transplanted into the testes of immunocompetent, prepubertal recipient animals, the recipients produced sperm carrying the donor haplotype and transmitted the donor genetic makeup to the offspring (illustrated in Fig. 1). This provided proof-of-principle that germ cell transplantation results in donor-derived sperm production and fertility also in a non-rodent species (Honaramooz *et al*., 2003b).

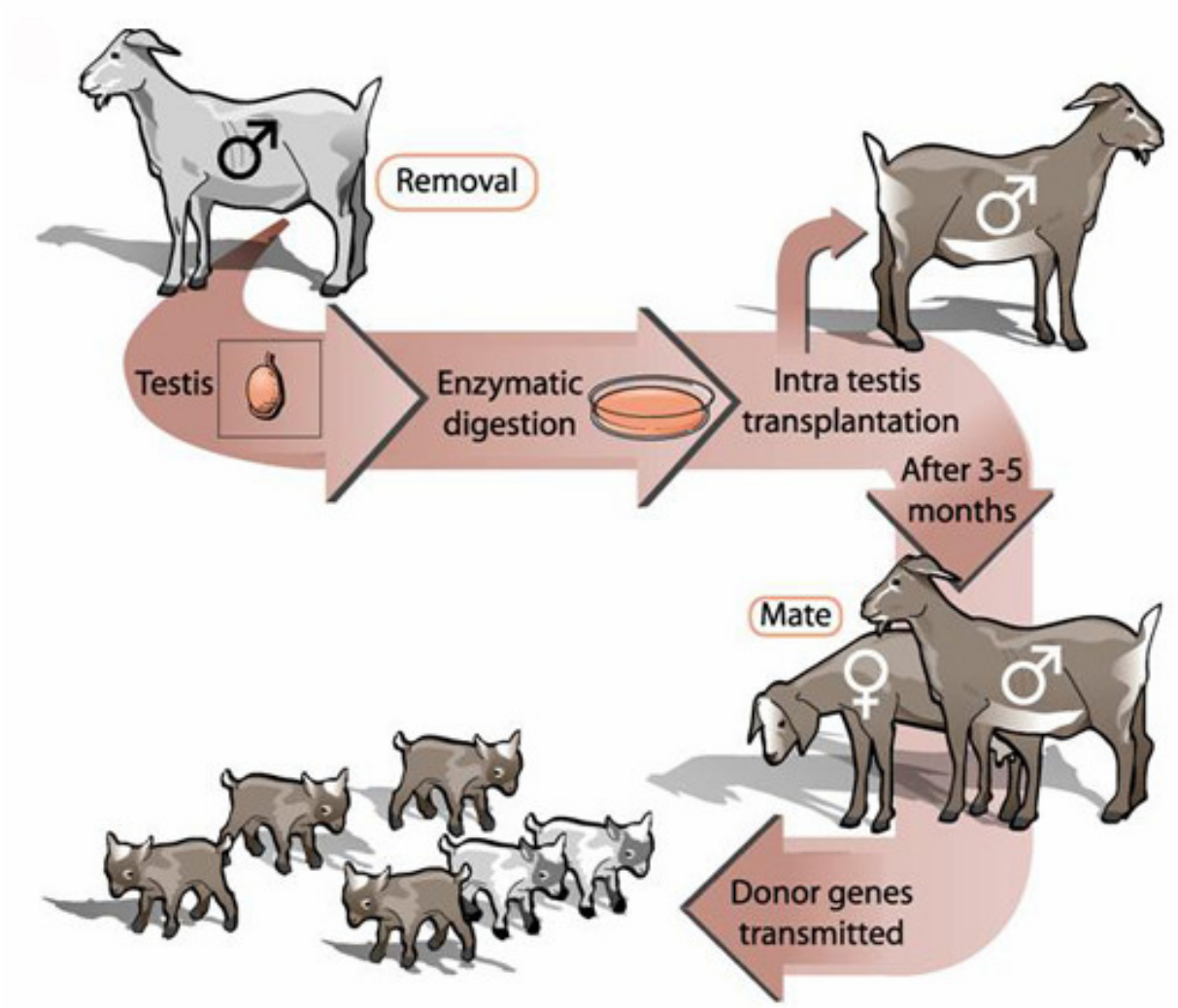

Figure 1. Schematic representation of germ cell transplantation in the goat (adapted from Honaramooz *et al.,* 2003b).

The success of germ cell transplantation requires the availability of a stem cell niche in the recipient testis. In rodents, the use of young mice and treating recipients with GnRH-agonists to suppress high intratesticular testosterone levels improved donor cell colonization (Shinohara *et al.*, 2001, Ogawa *et al.*, 1998; 1999a; Dobrinski *et al.*, 2001). Similarly, using prepubertal males as recipients has proven a successful strategy in germ cell transplantation in pigs, goats and cattle (Honaramooz *et al.*, 2002a , 2003b; Herrid *et al,* 2006). The efficiency of colonization of seminiferous tubules by the transplanted germ cells can be further improved 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). While its use in 2 adult boars has been described in combination with methylprednisolone to prevent thrombocytopenia,

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in utero treatment of pigs during a period of high proliferation of fetal gonocytes provides a more practical approach that resulted in depletion of germ cells with no observed adverse effects on the piglets or on sow health or fertility (Honaramooz *et al*., 2005). As an alternative to cytotoxic treatment, irradiation of the testes will also result in depletion of endogenous germ cells (Creemers *et al.*, 2002; Schlatt *et al*., 2002b). Provided a suitable radiation source is available, local testicular irradiation appears to be the method of choice in species where the anatomical position of the testes facilitates shielding of the body from irradiation, thereby minimizing systemic effects. Fractionated testicular irradiation resulted in depletion of germ cells in goats, rams and cats (Honaramooz *et al.*, 2005; Oatley *et al*., 2005; Kim *et al*., 2006). Interestingly, germ cell transplantation in rodents and perhaps also in older cattle requires that donor and recipients are closely related or that recipient animals are immuno-suppressed (Kanatsu-Shinohara *et al.*, 2003b; Zhang *et al*., 2003; Izadyar *et al*., 2004), whereas germ cell transplantation in pigs, goats and young bulls was successful also between unrelated individuals (Honaramooz *et al*., 2002b; 2003a; b; Mikkola *et al.*, 2006; Hill and Dobrinski, 2006; Herrid *et al.,* 2006). The testis is considered to be an immune privileged site, but it is unclear why transplantation between unrelated, immunocompetent animals is possible in domestic animal species but not in rodents. Nonetheless, this makes the technique infinitely more applicable in non-rodent species.

Transplantation of testis tissue

As discussed above, cross-species germ cell transplantation did not result in complete sperm production in species other than rodents, likely due to an incompatibility between donor germ cells and recipient testicular environment. Co-transplantation of the donor germ cells with their surrounding testicular tissue into a mouse host preserves the testicular environment and still allows experimentation in a small rodent. We therefore developed ectopic grafting of testicular tissue under the back skin of immunodeficient mice as an alternate approach for the maintenance and propagation of male germ cells that can be more readily applied to different mammalian species (Honaramooz *et al*., 2002b). Xenografting of testis tissue from newborn pigs and goats resulted, for the first time, in functional cross-species spermatogenesis from species other than rodents in a mouse host and sperm could be obtained from neonatal donors. To date, grafting of testis tissue from sexually immature males to immunodeficient mice has resulted in germ cell differentiation and production of sperm from a variety of different mammalian species (Table 1). Recently, we also established that adult human testis tissue can survive when grafted into host mice. While germ cell loss was noted in tissue with active spermatogenesis at the time of grafting, germ cells survived in tissue from patients with quiescent germinal epithelium (Schlatt *et al*., 2006; Geens *et al*., 2006).

Table 1. Testis tissue grafting: summary of results.

Donor	Spermatogenesis	Comments	References
Mouse (allograft)	complete	offspring	Schlatt *et al*., 2003
Hamster	complete		Schlatt *et al*., 2002a
Rabbit (not ectopic)	complete	offspring	Shinohara *et al*., 2002
Pig	complete	embryos	Honaramooz *et al*., 2002b
Goat, Sheep	complete	Sperm recovery	Honaramooz *et al*., 2002b; Dobrinski *et al*., 2003
Cattle	complete	inefficient	Oatley *et al*., 2004; Rathi *et al*., 2005
Cat	complete	Sperm recovery	Snedaker *et al*., 2004
Horse	complete	inefficient	Rathi *et al*., 2006
Rhesus monkey	complete	embryos	Honaramooz *et al*., 2004
Banteng	Meiotic cells		Honaramooz *et al*., 2005
Human (adult)	Germ cell survival		Schlatt *et al*., 2006; Geens *et al*., 2006

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Sperm recovered from allografts (mouse to mouse) and xenografts (monkey to mouse) supported embryo development when injected into oocytes (Schlatt *et al*., 2003; Honaramooz *et al*., 2004) and following embryo transfer, mouse sperm from allografts sired normal, fertile progeny (Schlatt *et al.*, 2003). The onset of spermatogenesis in xenografted pig testis tissue occurred slightly earlier than in the donor species (Honaramooz *et al*., 2002b) and testicular maturation and sperm production in rhesus macaque testis tissue was significantly accelerated by exposure to the endocrine environment of the castrated adult mouse host (Honaramooz *et al*., 2004). This shortened time to sperm production is due to accelerated maturation of the testicular somatic cells whereas the length of the spermatogenic cycle remains unchanged (Zeng *et al*., 2006b). Recently, we could also demonstrate that the global gene expression profile is very similar between testis tissue xenografts and testis tissue in situ (Zeng *et al.*, 2006a), further validating testis tissue xenografts as a representative model for mammalian spermatogenesis in the donor species.

While transplantation of testis tissue appears to be more easily applicable to different species than germ cell transplantation, it does not allow easy manipulation of selected cell types in the testis. We therefore exploited the morphogenic capacity of testis cells isolated from newborn animals to form seminiferous tubules de novo when transplanted ectopically in to host mice (Dufour *et al.*, 2002; Gassei *et al.*, 2006). Recently, we could demonstrate the remarkable ability of dissociated cells from neonatal porcine testis to regenerate functional testis tissue after ectopic transplantation to mouse hosts (Honaramooz *et al.*, 2006). This provides an accessible in vivo culture system that will now allow us to explore conditions necessary to recreate the essential cell associations to support spermatogenesis in vitro. Production of fertilization competent sperm from immature isolated germ cells in vitro would be an invaluable tool to study the molecular and cellular control of spermatogenesis and for the preservation of male fertility. Grafting of isolated testis cells provides a crucial first step in obtaining this so far elusive goal.

Applications of germ cell and testis tissue transplantation

Mouse spermatogonial stem cells can be cryopreserved for prolonged periods of time before transplantation, can still establish spermatogenesis in the recipient testis (Avarbock *et al.,* 1996) and live offspring resulted from spermatozoa produced after transplantation of frozen-thawed mouse germ cells (Kanatsu-Shinohara *et al*., 2003a). Therefore, germ cell transplantation could serve to restore male fertility after an insult to the testis. In cancer patients, germ cells could be frozen prior to irradiation or chemotherapy treatment for cancer as these treatments often lead to temporary or permanent destruction of spermatogenesis. Re-introduction of autologous germ cells could then restore fertility in the patient once the previous illness has been overcome. This approach was demonstrated in principle in the monkey (Schlatt *et al.*, 2002b) and its application in humans has been discussed (Radford *et al*., 1999, Radford, 2003; Brook *et al*., 2001), but so far no definitive data has been reported. Re-introduction of donor cells collected before treatment carries the risk that cancerous cells could also be re-introduced into the patient as demonstrated in leukemic rats (Jahnukainen *et al*., 2001). Cell sorting technology could potentially be employed to overcome this risk (Fujita *et al.*, 2005). Germ cell transplantation has an advantage over the cryopreservation of sperm prior to treatment, in that it could be applied to pre-pubertal males where sperm cannot be obtained or to adult males rendered azoospermic or teratozoospermic by the disease. The technique is also of great interest in domestic or endangered animals for its potential to preserve genetic material from immature males that are lost before they reach puberty (Pukazhenti *et al.*, 2006). Even when cryopreservation of sperm is possible from adult individuals, the preserved sperm will provide a finite resource. In contrast, cryopreserved germ cells will undergo genetic recombination after transplantation, thereby virtually preserving the entire genetic potential of the donor male. This will provide an invaluable advantage for application of germ cell transplantation to the conservation of genetic diversity.

Another important application of germ cell transplantation is transgenesis through the male germ line using transplantation of transfected germ cells. Transgenic mice and rats have been generated by viral transduction of germ cells prior to transplantation (Nagano *et al.*, 2001b; 2002b; Orwig *et al.*, 2002; Hamra *et al*., 2002). Recently, the advances in mouse germ cell culture even allowed for gene targeting and production of knock out mice using germ cell transplantation (Kanatsu-Shinohara *et al.*, 2006). This approach has tremendous potential in species where embryonic stem cell technology is not available and options to generate transgenic animals are inefficient. Current strategies to generate genetically modified large animals include pronuclear microinjection of DNA (Hammer *et al.*, 1985) and nuclear transfer technology using modified donor cells (Schnieke *et al.*, 1997; Cibelli *et al.*, 1998, Lai *et al.*, 2002; Park *et al.*, 2002; Chen *et al.*, 2002), 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 very valuable alternative. Introduction of a genetic modification prior to fertilization will circumvent problems associated with manipulation of gametes and early embryos and developmental

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abnormalities associated with nuclear reprogramming. In addition, even if embryonic stem cell technology becomes available, the time required until transgenic sperm can be harvested will be significantly shorter using germ cell transplantation (Fig. 2). While work in domestic animal models is still highly experimental, transgenesis through the male germ line is expected to provide an efficient alternative to currently existing technology for the introduction of genetic modifications in domestic animals.

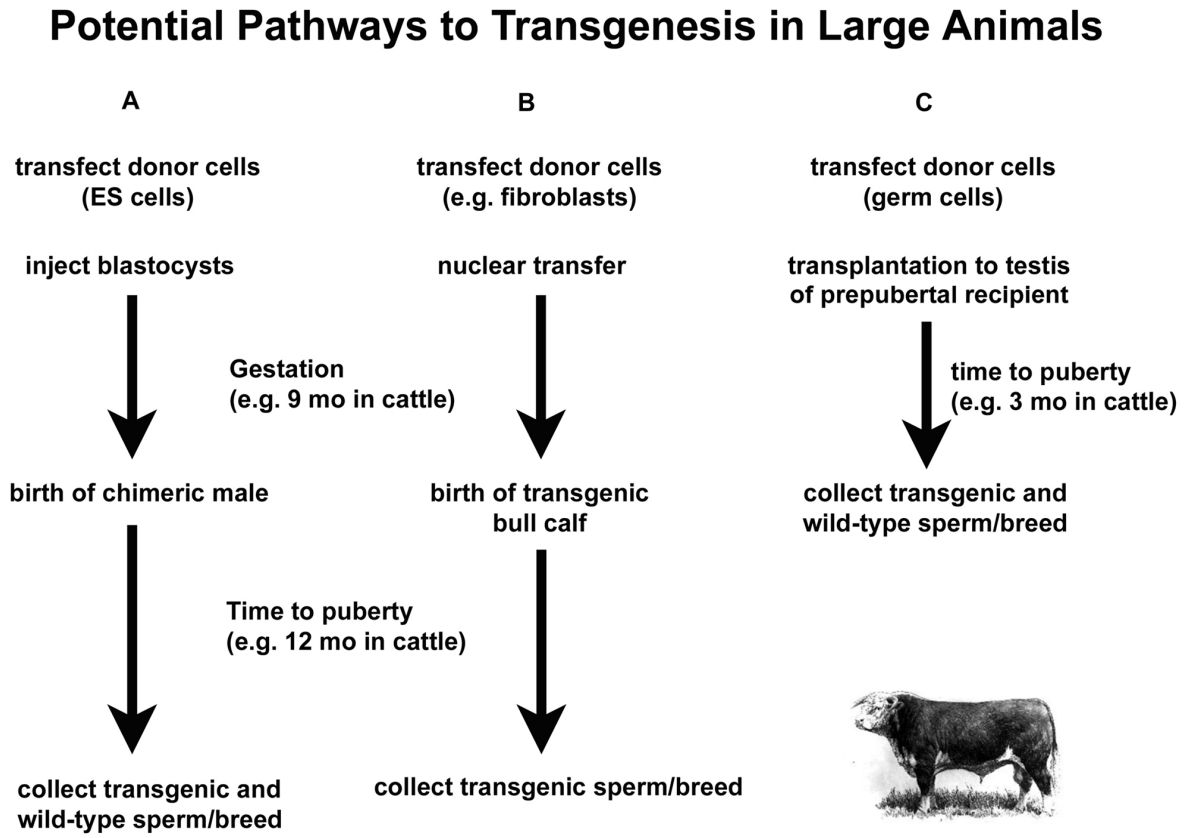

Figure 2. Comparison of three different approaches to transgenesis in large animals.
A: Transgene introduction through embryonic stem cells (Note: This has been adapted in principle from work in rodents as the technology is currently not available in large animals).
B: Transgenesis through somatic cell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

Transplantation of germ cells has tremendous potential for the preservation of fertility; however, it is technically difficult and not easily adapted to diverse species. In contrast, ectopic grafting of testis tissue to a mouse host is widely applicable to species ranging from mice to men. The accessibility of the tissue in the mouse host makes it possible to manipulate spermatogenesis and steroidogenesis in a controlled manner that is not feasible in the donor animal and certainly not in humans. This in turn will allow detailed analysis of the effects of toxins and compounds to enhance or suppress male fertility in an in vivo system without extensive experimentation in the target species (Jahnukainen *et al.*, 2006). Ectopic testis tissue grafting also represents a new option for male germ line preservation. Similar to isolated germ cells, testicular tissue can be stored frozen prior to grafting while retaining its developmental potential (Honaramooz *et al*., 2002b; Schlatt *et al*, 2002a). As it provides a source of male gametes even from immature gonads, grafting of fresh or preserved testis tissue offers an invaluable tool for the conservation of fertility. Spermatogenesis in testicular tissue formed de novo after rafting of isolated testis cells will further improve the versatility of the xenografting approach. Manipulation of specific pathways in germ cells or somatic cells prior to re-aggregation will provide a controlled, accessible system to study processes governing cell-cell interactions during testicular morphogenesis as well as spermatogenesis.

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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 large animals 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. Transplantation of germ cells and testis tissue are complimentary approaches to explore basic biological aspects of male germ line stem cells and testis function as well as potential causes of male infertility. Practical applications include the introduction of genetic modifications into the germ line of domestic animals, and the preservation of fertility in rare and endangered animals and in patients undergoing potentially sterilizing treatments for cancer therapy. Transplantation of germ cells or testis tissue will continue to significantly enhance our understanding of testis function and our ability to control and preserve male fertility.

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