Recent developments in testis tissue xenografting

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Abstract

Development of the mammalian testis and spermatogenesis involve complex processes of cell migration, proliferation, differentiation, and cell–cell interactions. Although our knowledge of these processes has increased in the last few decades, many aspects still remain unclear. The lack of suitable systems that allow to recapitulate and manipulate both testis development and spermatogenesis ex situ has limited our ability to study these processes. In the last few years, two observations suggested novel strategies that will improve our ability to study and manipulate mammalian spermatogenesis: i) testis tissue from immature animals transplanted ectopically into immunodeficient mice is able to respond to mouse gonadotropins and to initiate and complete differentiation to the level where fertilization-competent sperm are obtained, and ii) isolated testis cells are able to organize and rearrange into seminiferous cords that subsequently undergo complete development, including production of viable sperm. The current paper reviews recent advances that have been obtained with both techniques that represent novel opportunities to explore testis development and spermatogenesis in diverse mammalian species.


Introduction

Male fertility requires close, complex, and dynamic interactions of germ cells and supporting Sertoli cells in the epithelium of the seminiferous tubules. Organization of the seminiferous epithelium is a long process that starts early in life with the migration of primordial germ cells to the gonadal ridges of the embryo and the formation of seminiferous cords in conjunction with primitive Sertoli cells and peritubular myoid cells. After birth, the cords transform into tubules, and after a period of Sertoli cell proliferation germ cells actively divide and differentiate to give rise to the first wave of spermatogenesis. The male germ cells (spermatogonia, spermatocytes, and spermatids) are located in the seminiferous tubules in intimate association with somatic cells, in particular Sertoli cells, which form a simple columnar epithelium, resting on the basal lamina of the tubules and extending elaborate processes to surround the germ cells throughout the epithelium (Fig. 1). Surrounding peritubular myoid cells located at the extratubular side of the basal lamina contribute to the wall of the tubules (Orth 1993).

Testis development and spermatogenesis are regulated by numerous factors and although our knowledge of them has increased significantly, it is still far from being complete, particularly in postnatal development and in non-rodent species (Merchant-Larios & Moreno-Mendoza 2001). In the last few decades, important discoveries have provided new alternatives with unprecedented opportunities for the study of testis development and spermatogenesis. Germ cell transplantation in the rodent model has proven to be an invaluable tool for the study of spermatogenesis and spermatogonial stem cell biology (Brinster & Avarbock 1994, Brinster & Zimmermann 1994, Brinster 2007). The technique has now also been successfully established in pigs (Honaramooz et al. 2002a), goats (Honaramooz et al. 2003a, 2003b, 2008b), cattle (Izadyar et al. 2003, Herrid et al. 2006), monkeys (Schlatt et al. 1999, 2002a) sheep (Rodriguez-Sosa et al. 2006, 2009), and dogs (Kim et al. 2008); and although its use in these species is now a realistic option, further studies are needed before this approach becomes more widely applicable (Dobrinski & Travis 2007). One current limitation is that germ cell transplantation to study spermatogenesis in these species and to manipulate their germ cells is logistically difficult and expensive. Therefore, novel alternatives that allow procedures to be performed without extensive experimentation in the target species are highly desirable. Transplantation of germ cells from donors of larger species into mouse testes results in incomplete spermatogenesis, with no differentiation beyond the spermatogonial stage. This is presumably due to an incompatibility between the donor germ cells and the microenvironment of the mouse testis.
Honaramooz exhibits both endocrine and spermatogenic functions rearrange to generate complete testis tissue that males transplanted under the dorsal skin of mice testicular somatic cells and germ cells from neonatal in situ development to host gonadotropins and developed similarly to transplanted ectopically to mice were able to respond that testis pieces from immature domestic males mentation in the rodent host. First, it was reported necessary compatibility, yet still allows experi-
sation of the donor germ cells with their surrounding somatic compartment into the rodent host preserves the necessary compartment therefore seems to be necessary for germ cells to proliferate and differentiate. Recently, two important discoveries have shown that co-transplantation of the donor germ cells with their surrounding somatic compartment into the rodent host preserves the necessary compatibility, yet still allows experimenta-
tion in the rodent host. First, it was reported that testis pieces from immature domestic males transplanted ectopically to mice were able to respond to host gonadotropins and developed similarly to development in situ, including the formation of fertilization competent sperm (Honaramooz et al. 2002b). More recently, it was reported that isolated testicular somatic cells and germ cells from neonatal males transplanted under the dorsal skin of mice rearrange to generate complete testis tissue that exhibits both endocrine and spermatogenic functions (Honaramooz et al. 2007, Kita et al. 2007). Whereas transplantation of testis cells mimics the complete development process, transplantation of tissue recapitulates the postnatal phase. These strategies are technically much easier than spermatogonial transplanta-
tion in larger animals for manipulation and study of testis development and spermatogenesis. Since it has been shown that sperm obtained from testis tissue xenografts can be used for ICSI to produce embryos and offspring (Honaramooz et al. 2002a, 2002b, 2004, 2008a, Schlatt et al. 2003), these strategies also represent new approaches for preserving the germ line of valuable males or endangered species (Dobrinski 2005, Paris & Schlatt 2007). Here, we review some of the more recent advances accomplished with ectopic transplantation of tissue and cells from larger species into immuno-
deficient mice.

Xenotransplantation of testis tissue
At first glance, the testis does not appear to be a suitable tissue for grafting because of the complexity of the seminiferous epithelium and the architecture of its vascular and duct systems. However, trans-
plantation of testicular tissue has been performed since 1924, and has provided important insights into testicular function. Autologous and homologous transplantation of testicular tissue have been reviewed by Gosden & Aubard (1996a, 1996b). Immune-deficient lines of mice now allow xenotransplantation of testicular and other tissues (Paris et al. 2004), which makes it possible to transplant tissue from large animals into mouse hosts (Dobrinski 2005). Xenotransplantation of testicular tissue (human fetal testis into the abdominal wall of adult nude mice) was first performed in 1974 (Skakkebaek et al. 1974). Subsequently Hochereau-de-Reviers & Perreau (1997) transplanted ovine fetal testis into the scrotum of intact nude mice and reported differentiation of gonocytes into spermatogonia and primary spermatocytes. However, complete cross species spermatoge-
 genesis was first reported in 2002 (Honaramooz et al. 2002b). In that report, pieces of testis tissue from newborn pigs and goats were able to survive and displayed complete development with production of sperm. Since then, testis tissue xenografting has been tested in numerous species. In most studies, the testicular tissue that is transplanted is small cubes of \(0.5–1\text{ mm}^3\), 3–5 mg, or 10 mg (Honaramooz et al. 2002b, Schlatt et al. 2002b, Schmidt et al. 2006a, 2006b). These tissue fragments are transplanted into multiple sites under the dorsal skin on either side of the spinal cord, 2–8 pieces/mouse. The recipients are immune-deficient mice, usually nude (T-cell deficient) mice (Honaramooz et al. 2002b, Schlatt et al. 2002b, Oatley et al. 2004, 2005, Rathi et al. 2005, 2006, Zeng et al. 2006), but severe-combined immuno-
deficient mice (T- and B-cell deficient mice) have also been used (Honaramooz et al. 2004, Snedaker et al. 2004, Rathi et al. 2005, 2006, Schlatt et al. 2006). No difference has been found between xenografts transplanted into these two strains (Rathi et al. 2005, 2006, Geens et al. 2006). The recipients are castrated prior to or during the transplantation surgery. With the exception of the marmoset (Schlatt et al. 2002b, Wistuba et al. 2004), testicular grafts from all species evaluated so far are able to survive and respond to mouse gonadotropins once they are transplanted under the dorsal skin of castrated mice. This response is characterized by the development of immature xenografts, and the production of spermatozoa (Fig. 2).
and androgens. Several factors affect testis tissue survival and function after transplantation. So far, the best understood are the effects of donor species and of the age and developmental stage of the donor.

**Effects of donor species**

Testis tissue xenografting has been evaluated in numerous species with variable results. Three major variables have been affected by the species: development through spermatogenesis; timing of spermatogenic development; and efficiency of spermatogenesis (Table 1). Regarding the first variable, testis tissue from species phylogenetically distant from mice such as rhesus monkeys, cattle, goats, cats, and pigs is responsive to host gonadotrophins as demonstrated by spermatogenesis after grafting. A notable exception is the marmoset, which appears insensitive to mouse LH due to a deletion in exon 10 of its LH-receptor gene. This blocks androgen production and results in poor spermatogenesis after grafting (Schlatt et al. 2002b, Wistuba et al. 2004).

Shortening of the time required until first appearance of testicular sperm in immature testicular xenografts is evident in species like pig and monkey, and it is attributed to the capacity of transplanted tissue to immediately respond to the gonadotropins of the castrated adult recipient (Honaramooz et al. 2002b, 2004). Two notable exceptions are xenografts of testes from cattle and cats. In cattle, onset of spermatogenesis is slightly advanced or similar to testes in situ (Oatley et al. 2004, 2005, Rathi et al. 2005). In cats, this timing is delayed (Snedaker et al. 2004, Kim et al. 2007). Interestingly, similar to observations in other donor species, bovine and feline xenografts start developing earlier than in age-matched in situ controls, with a slight acceleration of the onset of meiosis. However, bovine germ cells seem to arrest at meiosis resulting in slight or no advance (Rathi et al. 2005). In cats, delay in the timing of testicular maturation may be controlled by the testis (Snedaker et al. 2004) or may be a result of an incompetence of the testis tissue to support full spermatogenesis for some time, suggesting an immaturity of some component within the feline testis xenograft (Kim et al. 2007).

The efficiency of spermatogenesis in xenografts is also species dependent. While the number of spermatozoa produced by pig and goat testicular xenografts was similar to that produced in normal testes on a ‘per gram of tissue’ basis (Honaramooz et al. 2002b), complete spermatogenesis does not occur in all seminiferous tubules in xenografts of cattle (Oatley et al. 2004, 2005, Rathi et al. 2005, Schmidt et al. 2006a, 2006b), horses (Rathi et al. 2006), cats (Snedaker et al. 2004, Kim et al. 2007), sheep (Zeng et al. 2006, Arregui et al. 2007), and marmosets (Schlatt et al. 2002b, Wistuba et al. 2004).
et al. 2008a), and rhesus monkeys (Honaramooz et al. 2004, Rathi et al. 2008). One potential reason for failure to achieve complete spermatogenic differentiation appears to be incomplete Sertoli cell maturation in the grafted tissue (Rathi et al. 2008).

**Age and developmental stage of donor**

The second most explored factor affecting testis xenograft survival and development is the age or developmental stage of the donor. Survival of xenografts decreases with the degree of maturity of the donor tissue. Tissue from adult donors shows poor survival and a marked tendency to degenerate making it unsuitable for transplantation (Schlatt et al. 2002b, Geens et al. 2006, Kim et al. 2007, Arregui et al. 2008b). Some spermatooza were produced from transplanted adult mouse and human testicular tissue. However, these spermatooza were believed to be from differentiating germ cells that completed spermatogenesis after grafting, rather than arising de novo from spermatogonial stem cells (Schlatt et al. 2002b, 2006, Geens et al. 2006). Degeneration of adult testis xenografts seems to occur faster in those species with higher spermatogenic activity such as pig and goat, and even in a particular species, degeneration is more severe in mature adult donors than in subadult donors (Arregui et al. 2008b). Several reasons have been proposed to explain poor survival of adult testis tissue, including lack of proliferation of Sertoli cells, increased sensitivity to ischemia, and a decreased angiogenic ability of the adult tissue (Schlatt et al. 2002b, Arregui et al. 2008a, 2008b).

Immature tissue shows the best survival. Tissue from neonatal and prepubertal donors displays better survival and support of spermatogenesis than that from donors in which maturation has been initiated. It appears that once meiosis has occurred consistently throughout the donor tissue, the ability of that tissue to survive as a xenograft and support spermatogenesis declines dramatically (Rathi et al. 2006, Kim et al. 2007). Ischemic damage is expected to occur in any type of transplantation, and an initial loss of germ cells has been described (Rathi et al. 2006). Recovery of germ cell numbers and establishment of spermatogenesis in testis xenografts must occur by proliferation and differentiation of spermatogonial stem cells (Rathi et al. 2006, Huang et al. 2008). Differences in stem cell cohorts between prepubertal and pubertal donors may contribute to differences in their ability to survive and support spermatogenesis. Moreover, it is also possible that the somatic cell components and later stages of germ cells contribute to this difference (Kim et al. 2007). By the time meiosis has started, Sertoli cells have matured and their proliferative activity decreases (Meachem et al. 2005), and this may contribute to a decreased ability of pubertal donors to replenish Sertoli cells lost after transplantation. On the other hand, meiotic and postmeiotic germ cells may be less likely to survive hypoxia after transplantation due to their dependence on oxidative metabolism (Rathi et al. 2006, Kim et al. 2007).

**Effects of recipient**

Most xenotransplantations have been into the subcru- ciate tissue on the dorsal surface of the rodent host, although Shinohara et al. (2002) successfully transplanted immature rabbit testicular tissue into the testes of mice. There are differences in temperature and

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**Table 1 Development of testis tissue and spermatogenesis in ectopic testis xenografts.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Donor age</th>
<th>% Tubules with complete spermatogenesis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Collection time&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Onset of spermatogenesis&lt;sup&gt;c&lt;/sup&gt;</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td>1–2 weeks</td>
<td>52 (7–98)</td>
<td>7–8 months</td>
<td>Advanced</td>
<td>Honaramooz et al. (2002b) and Zeng et al., (2006, 2007)</td>
</tr>
<tr>
<td>Cattle</td>
<td>1–8 weeks</td>
<td>&lt;15</td>
<td>24–36 weeks</td>
<td>Similar or slightly advanced</td>
<td>Honaramooz et al. (2002b) and Zeng et al. (2006) and Arregui et al. (2008a)</td>
</tr>
<tr>
<td>Goat</td>
<td>4 weeks</td>
<td>Not mentioned</td>
<td>12–28 weeks</td>
<td>Not determined</td>
<td>Honaramooz et al. (2002b)</td>
</tr>
<tr>
<td>Sheep</td>
<td>1–2 weeks</td>
<td>64 (2–92)</td>
<td>12–28 weeks</td>
<td>Similar or advanced</td>
<td>Honaramooz et al. (2002b) and Zeng et al. (2006) and Arregui et al. (2008a)</td>
</tr>
<tr>
<td>Horse</td>
<td>2 weeks–4 years</td>
<td>~5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8 months</td>
<td>Not determined</td>
<td>Advanced</td>
</tr>
<tr>
<td>Cat</td>
<td>1 week–15 months</td>
<td>6–25&lt;sup&gt;e&lt;/sup&gt;</td>
<td>35–50 weeks</td>
<td>Advanced</td>
<td>Honaramooz et al. (2004) and Rathi et al. (2008)</td>
</tr>
<tr>
<td>Rhesus macaque</td>
<td>3–13 months</td>
<td>4 (0–15)</td>
<td>7 months</td>
<td>Advanced</td>
<td>Honaramooz et al. (2002b) and Schlatt et al. (2003)</td>
</tr>
<tr>
<td>Human</td>
<td>Adult</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>Geens et al. (2006) and Schlatt et al. (2006)</td>
</tr>
<tr>
<td>Mouse&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1–2 days</td>
<td>~10–20</td>
<td>4–16 weeks</td>
<td>Similar</td>
<td>Honaramooz et al. (2002b) and Schlatt et al. (2003)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Presence of elongated spermatids and/or sperm. <sup>b</sup>Collection time at which full spermatogenesis was observed. <sup>c</sup>In comparison with testis in situ. <sup>d</sup>Only in a 10-month-old donor. <sup>e</sup>In donors younger than 7 months of age. <sup>f</sup>Allografting, included for comparison.
vascularity between these sites that would be expected to affect graft development and survival. Homologous transplantsations in rats by Turner (1938) showed that the anterior chamber of the eye provided a better environment for testicular grafts than subcutaneous, intraperitoneal, intramuscular, and scrotal sites as measured by percentage survival of tissue and sperm production.

Most xenotransplantation studies have used castrated males as recipients. This avoids interference of the host testis in response of xenografts to host gonadotropins, and allows androgen production by graft Leydig cells to be monitored based on the weight and histology of the host seminal vesicles, the development of which is androgen dependent (Gosden & Aubard 1996b). In addition, removal of the host testes releases the negative feedback on the mouse pituitary secretion of FSH, resulting in increased levels of FSH at the time of grafting (Schlatt et al. 2003). These increased gonadotropin levels are thought to support Sertoli cell proliferation and graft development until a feedback axis is re-established between the grafted tissue and the host hypothalamus and pituitary. Already in 1938, Turner found that survival between the grafted tissue and the host hypothalamus development generally is improved in castrated mouse males as recipients. This avoids interference of the host serum with Sertoli cell proliferation and differentiation at the time of grafting (Honaramooz et al. 2006). However, it remained to be demonstrated whether i) seminiferous tubules generated from isolated cells could form a functional stem cell niche, ii) germ cells could locate in this niche, and iii) interact with it to support complete spermatogenesis. To answer these questions, concentrated pellets from single-cell suspensions prepared from neonatal porcine testes, composed of ~50% Sertoli cells, 5% germ cells, 20% peritubular myoid cells, and 10% Leydig cells, were transplanted under the dorsal skin of immunodeficient mice. After a few days, cells had rearranged into cord-like structures, and by 4 weeks cords transformed into tubules. By that time, germ cells contained in the tubules had migrated to the basal lamina and started to proliferate. After 30 weeks, complete spermatogenesis had occurred, and sperm were present in the lumen of ~10% of the tubules (Honaramooz et al. 2007).

Similarly, de novo morphogenesis of testis tissue was also described when isolated testis cells from embryonic or neonatal mouse and rat were grafted ectopically to mouse hosts (Kita et al. 2007). In that study, isolated cells from newborn testes were mixed with cultured mouse germ line stem cells carrying a GFP marker and grafted into mouse hosts. In the reconstituted tissue, spermatogenesis progressed to the stage of round spermatids derived from the cultured cells. ICSI of these cells into mouse oocytes and embryo transfer resulted in the birth of normal pups expressing the GFP marker, demonstrating that haploid cells formed in the reconstituted testis tissue were capable to support complete development (Kita et al. 2007).

In a recent study in sheep, a heterogeneous cell suspension obtained from 2-week-old lambs was concentrated and transplanted under the dorsal skin of nude mice. Similar to pig testis morphogenesis, by 4 weeks after grafting lamb testis the cells had organized into tubules. No differentiated germ cells were observed 4, 8, 12, and 16 weeks after transplantation. However, at 35 weeks, some tubules contained pachytene spermatocytes, and by 40 weeks after grafting, complete spermatogenesis had occurred (Arregui et al. 2008a). Taken together, these studies demonstrated the amazing capability of isolated postnatal testis cells to recapitulate testis development, rearrange into seminiferous cords, and undergo complete differentiation. Recently, the ability to form seminiferous tubules from isolated Sertoli cells after grafting into mouse hosts has also been demonstrated for bovine Sertoli cells (Zhang et al. 2008), and has been applied to the study of Sertoli cell function in the rat (Gassei et al. 2008).

Xenografting of testis tissue into mice

While testis tissue xenografting allows the maintenance of the integrity of the seminiferous epithelium, it does not allow easy manipulation of selected cell types in the testis. The morphogenic ability of isolated testis cells to reconstitute functional testis tissue and support spermatogenesis after transplantation opens new possibilities to accomplish that aim. Grafting of isolated testis cells has been developed more recently, and as such it has been explored less than testis tissue xenografting. Although the factors that affect testis tissue (reviewed above) may presumably have a similar effect on testis cells after transplantation, additional research is necessary to evaluate those effects. So far, this technique has successfully been evaluated with cells from newborn donors in pigs (Honaramooz et al. 2007), rodents (Kita et al. 2007), and sheep (Arregui et al. 2008a).

Formation of functional testis tissue from transplantation of isolated cells built on previous reports showing that co-transplantation of porcine Sertoli cells and pancreatic islets, to confer immunoprotection by Sertoli cells, resulted in cord formation by Sertoli and peritubular myoid cells (Kin et al. 2002). Similarly, isolated rat testis cells transplanted under the kidney capsule of mice or maintained in culture and then transplanted ectopically had the ability to generate seminiferous cords (Dufour et al. 2002, Gassei et al. 2006). However, it remained to be demonstrated whether i) seminiferous tubules generated from isolated cells could form a functional stem cell niche, ii) germ cells could locate in this niche, and iii) interact with it to support complete spermatogenesis. To answer these questions, concentrated pellets from single-cell suspensions prepared from neonatal porcine testes, composed of ~50% Sertoli cells, 5% germ cells, 20% peritubular myoid cells, and 10% Leydig cells, were transplanted under the dorsal skin of immunodeficient mice. After a few days, cells had rearranged into cord-like structures, and by 4 weeks cords transformed into tubules. By that time, germ cells contained in the tubules had migrated to the basal lamina and started to proliferate. After 30 weeks, complete spermatogenesis had occurred, and sperm were present in the lumen of ~10% of the tubules (Honaramooz et al. 2007).

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Applications of testis tissue and testis cell transplantation

Ectopic testis tissue grafting 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. 2002b). Moreover, testis tissue xenografting allows experimentation in small rodents while minimizing the number of larger animal donors required and allowing replication of treatments within donor to eliminate a potential donor effect (Rathi et al. 2008). It has been demonstrated that pig testis xenografts have a gene expression profile similar to that of testis tissue in situ (Zeng et al. 2007), and that the cycle of the seminiferous epithelium is conserved in pig and sheep testes xenografts (Zeng et al. 2006), supporting the equivalence of xenograft development and spermatogenesis to those of the normal tissue in situ tissue. Currently, grafting of fresh or preserved testis tissue provides the only technique to obtain male gametes from immature donors, thereby offering an invaluable tool for the conservation of fertility in rare or endangered species (Dobrinski & Travis 2007), neonatally lethal rodent models (Ohta & Wakayama 2005, Naughton et al. 2006), and potentially even juvenile cancer patients (Orwig & Schlatt 2005). Spermatogenesis in testicular tissue formed de novo after grafting of isolated testis cells will further improve the versatility of the xenografting approach. Potential applications (Fig. 3) include co-grafting of germ cells and somatic cells from different donors to define the cellular origin of a given spermatogenic defect, e.g. germ cells from aged donor grafted with somatic cells from young donor, or germ cells from affected donor with somatic cells from wild-type donor and vice versa. 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.

Conclusions and final remarks

Many aspects of testis development and spermatogenesis remain to be elucidated due mainly to a lack of systems that allow recapitulation and easy manipulation of these complex processes ex situ. In the last years,
transplantation of testis tissue and isolated cells into immunodeficient mouse hosts has provided novel strategies to explore testis development and spermatogenesis. To date, ectopic grafting of testis tissue has been explored more extensively than ectopic transplantation of isolated testis cells. Factors affecting the survival and development of grafted testis tissue have been identified with the donor species and the age or developmental stage of the donors the most studied so far. Testis cell transplantation is a newer approach that will extend applicability and potential of xenografting to preserve fertility and to study and manipulate spermatogenesis in a variety of mammalian species.

Declaration of interest
The authors declare that there is no conflict of interest.

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