THE STUDY AND APPLICATION OF

TESTIS TISSUE XENOGRAFTING

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TITLE OF THESIS: The Study and Application of Testis Tissue Xenografting

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ABSTRACT

Testis tissue xenografting (TTX) provides a novel *in vivo* model for the study of testis function, and a previously-unavailable opportunity to produce spermatozoa in the grafts from immature donors of diverse species. The overall objectives of this thesis were to examine a number of factors that potentially affect the outcome of TTX, and to apply TTX using immature bison and deer donors as models for endangered ungulates.

The objective of the first experiment was to examine the effects of recipient mouse strain, gender and gonadal status on the outcome of TTX. Eight small fragments of neonatal porcine testis tissue (~5 mg each) were grafted under the back skin of immunodeficient mice of different strains (SCID *vs.* nude), gender (male *vs.* female), and gonadal status (intact *vs.* gonadectomised), using a 2×2×2 factorial design (8 groups, n = 7 mice/group). The xenografts were recovered at 8 mo post-grafting and evaluated for gross and histological attributes. Gonadectomy of the recipients did not affect any of the measured outcomes of TTX ($P > 0.05$), and data were pooled into four groups based on recipient strain and gender. Overall, male recipient mice had grafts with higher mean (+SEM) recovery rate (97 ± 2.3% *vs.* 88 ± 2.4%, $P = 0.004$), weight (348 ± 26.3 *vs.* 104 ± 27.0 mg, $P < 0.001$), seminiferous tubular diameter (150 ± 3.3 *vs.* 108 ± 5.3 mg, $P < 0.001$), percentage of tubules containing spermatozoa (32 ± 3.2 *vs.* 6 ± 1.8%, $P < 0.001$), elongated spermatids (13 ± 1.4% *vs.* 4 ± 0.8%, $P < 0.001$), and round spermatids (10 ± 1.2% *vs.* 6 ± 1.1%, $P = 0.006$) than female mice. Overall, SCID mice had grafts with higher recovery rate (98 ± 2.4% *vs.* 87 ± 2.3%, $P = 0.001$), average weight (292 ± 27.0 *vs.* 160 ± 26.3 mg, $P = 0.001$), tubular density (44 ± 3.3 *vs.* 33 ± 2.1, $P = 0.02$), percentage of tubular cross-sections containing spermatocytes (27 ± 3.7% *vs.* 13 ± 2.3%,

P = 0.003) than nude mice. Among the four groups of recipients, the grafts from male SCID mice had the highest weight ($P < 0.05$) and percentage of tubules containing spermatozoa ($P < 0.05$).

The objective of the second experiment was to evaluate the effect of using different numbers of donor testis tissue fragments on the outcome of TTX. Fragments of donor piglet testis tissue were grafted subcutaneously under the back skin of four groups of castrated male nude mice (n = 10/group). Each group of recipient mice received 2, 4, 8, or 16 fragments per mouse. Mice were sacrificed at 8 mo post-grafting, and xenografts were evaluated for physical growth and histological development. The relative weight of the vesicular gland (index) was also determined as a measure of bioactive androgen production by grafts in castrated recipient mice. The overall graft recovery rate was ~94% (range 86-98%) which did not differ among the groups ($P > 0.05$). The group of mice that received 16 testis tissue fragments had higher mean (+ SEM) graft weights (278 ± 39.4 *vs.* 106 ± 38.0, $P = 0.02$), total graft weight ($2{,}443 \pm 338.8$ *vs.* 192 ± 76.2, $P < 0.001$), vesicular gland index (0.5 ± 0.06 *vs.* 0.1 ± 0.06, $P = 0.007$), and percentage of seminiferous tubules with round spermatids (11 ± 1.5 *vs.* 3 ± 1.3, $P = 0.03$) than the group of mice that received two testis tissue fragments.

The objective of the third experiment was to assess the use to salvage testis tissue from neonatal/immature bison or deer donors using TTX into immunodeficient recipient mice as models for closely-related rare or endangered ungulates. Donor testis tissue fragments from two newborn bison calves (*Bison bison bison*) and a 2-mo-old white-tailed deer fawn (*Odocoileus virginianus*) were grafted under the back skin of gonadectomised nude mice (n = 15 and n = 7 for bison and deer groups, respectively, 8 testis

fragments/mouse). To examine the potential effect of individual donors, we grafted four testis tissue fragments from one bison calf on one side of the recipient and four fragments from the second bison calf on the other side. Single grafts were surgically removed from representative recipient mice every 2 mo for up to 16- and 14 mo post-grafting, for bison and deer groups, respectively. The overall graft recovery rates were 69% and 63% for bison and deer groups, respectively. For bison grafts, a donor effect on efficiency of spermatogenesis was also observed. The weight of bison testis tissue xenografts increased ($P < 0.02$) ~4-fold by 2 mo and ~10-fold by 16 mo post-grafting, and gradual maturational changes were evident in the form of seminiferous tubule expansion starting at 2 mo, first appearance of spermatocytes at 6 mo, round spermatids at 12 mo, and elongated spermatids at 16 mo post-grafting. Testis tissue xenografts from donor white-tailed deer also showed a gradual development starting with tubular expansion by 2 mo and presence of spermatocytes by 6 mo post-grafting, round and elongated spermatids by 8 mo, followed by fully-formed spermatozoa by 12 mo post-grafting. The timing of complete spermatogenesis roughly corresponded to the reported timing of sexual maturation in these species.

Taken together, the findings in this thesis suggest that male SCID mice provide a more suitable recipient model for TTX with neonatal porcine testis tissue; recipient mice can be grafted with as many as 16 testis tissue fragments for optimal results; and that TTX is a feasible strategy for salvaging genetic materials from immature males of rare or endangered ungulates that die prematurely.

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LIST OF ABBREVIATIONS

µm	Micrometer(s)
ANOVA	Analysis of variance
A_s	A_{single}
BMP4	Bone morphogenetic protein 4
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate buffered saline
dpc	Days post coitum
FGF2	Fibroblast growth factor 2
G	Gram(s)
GDNF	Glial cell line-derived neurotrophic factor
ICSI	Intracytoplasmic spermatozoa injection
IUCN	International Union for Conservation of Nature
LH	Luteinizing hormone
Mg	Milligram(s)
mm^2	Square millimetre(s)
mm^3	Cubic millimetre(s)
mo	Month(s)
NK cells	Natural killer cells
NOG	NOD/Shi-SCID IL-2Rγc^{null}
PGC	Primordial germ cell
Prkdc	Protein kinase, DNA activated, catalytic polypeptide
r	Pearson's correlation coefficients
SEM	Standard error of mean
SSC	Spermatogonial stem cell

SRY	Sex determining region Y
SCID	Severe combined immunodeficiency
TTX	Testis tissue xenografting
wk	Week(s)

CHAPTER 1: GENERAL INTRODUCTION

Mammalian spermatogenesis is a complex cyclic process that results in production of spermatozoa capable of fertilizing the oocyte (Russell *et al.* 1990). The study of testicular development, maturation, and spermatogenesis has largely been limited to animal experimentation and frequently focused on laboratory rodents as models for humans. Information on testis function in livestock species is relatively limited.

Although our knowledge of spermatogenesis has advanced considerably in recent years, numerous questions remain unanswered due to the lack of proper *in vivo* or *in vitro* models (de Rooij and Russell 2000). Recently, testis tissue xenografting (TTX) was introduced as a novel *in vivo* model for the study of testis function (Honaramooz *et al.* 2002). This study represented the first report of successful cross-species induction of complete spermatogenesis and steroidogenesis in a recipient mouse model. In TTX, small fragments of testis tissue from immature mammalian donors are grafted subcutaneously under the back skin of immunodeficient recipient mice. When given sufficient time, the xenografted testis tissue underwent physical growth (up to 100-fold), and maturation, and eventually produced donor-derived spermatozoa.

Previous reports of cross-species transplantation of isolated testis cells from non-rodent species into immunodeficient mice did not result in complete spermatogenesis (Dobrinski *et al.* 1999; 2000; Nagano *et al.* 2001; 2002). On the other hand, TTX has been successfully applied using a variety of donor species including immature mice, rats, hamsters, cats, dogs, pigs, goats, sheep, cattle, horses, monkeys, and humans into

immunodeficient mice (reviewed in Rodriguez-Sosa and Dobrinski 2009; Abrishami *et al.* 2010a). The key in the success of TTX is maintaining the complex structural integrity of the testis tissue and allowing the testicular somatic and germ cells to maintain normal interactions (Honaramooz *et al.* 2002, Schlatt *et al.* 2003, Dobrinski and Rathi 2008).

The spermatozoa retrieved from testis tissue xenografts showed the typical characteristics of testicular spermatozoa of the donor species, and following intracytoplasmic spermatozoa injection (ICSI), fertilized the oocyte and supported development to result in viable offspring (Honaramooz *et al.* 2002; Schlatt *et al.* 2003; Honaramooz *et al.* 2008; Nakai *et al.* 2010).

There are a number of potential applications for TTX. It provides a previously-unavailable system through which the developmental potential of a donor testis can be examined. It also makes it possible to study spermatogenesis and timing of testicular maturation in species such as livestock or primates where *in situ* studies are difficult or impossible. Additionally, this strategy may offers a method for experimental production of genetically-modified spermatozoa (by inserting genes of interest into the tissue *in vitro* prior to TTX), and for preservation of fertility potential of prepubertal boys undergoing sterilizing cancer treatment. Furthermore, it can provide an alternative approach for the preservation of genetic material from rare or endangered animals that die before they are mature.

As expected, the spermatogenic efficiency of TTX varies among reports from different research groups, depending on the donor species or developmental stage of the donor tissue. However, the success of TTX also varied when these conditions were kept

constant (Rathi *et al.* 2005; 2006; Zeng *et al.* 2006; Abrishami *et al.* 2010a). To optimise TTX for the study of testis function or for production of spermatozoa for assisted reproductive technologies, different aspects of the technique including those related to the donor tissue and recipient mice need to be thoroughly evaluated. To date, no study has comprehensively investigated the effect of donor tissue mass or recipient mouse characteristics on the outcomes of TTX.

Conservation of rare or endangered species through preservation of the germline and genetic diversity is one of the most challenging tasks of the reproductive biologists. Although procedures are in place for retrieval and cryopreservation of ejaculated, epididymal or testicular spermatozoa, even shortly after death of an individual, they are not applicable to sexually immature males (Kishikawa *et al.* 1999; Gañán *et al.* 2009; Martínez *et al.* 2008). The fact that TTX provides a unique *in vivo* culture system for maturation of the donor testis tissue and production of spermatozoa from immature animals that die prematurely is ideal for conservation purposes. However, TTX has not been previously applied in rare or endangered species and preliminary studies using readily-available donor tissue from non-domestic species is required before it can be applied in closely-related wild rare or endangered species.

Therefore, the first objective of this study was to determine which combination of recipient factors including the mouse strain, gender or gonadal status will provide the best model for TTX. As a second objective and as part of our goal to improve the efficiency of TTX, we also investigated the suitable number of testis tissue fragments which can be grafted per mouse. Our third objective was to expand the use of this novel

technique to the study of spermatogenesis in non-domestic donor species. Therefore, we evaluated the long-term outcome of TTX from specialised hoofstock donors (bison and white-tailed deer) into recipient immunodeficient mice.

CHAPTER 2: LITERATURE REVIEW

I. TESTIS TISSUE AND ITS XENOGRAFTING

2.1 Adult seminiferous epithelium

The testis, as the male gonad, produces steroid hormones and male gametes. The parenchyma is the functional unit of the testis and is made up of seminiferous tubules and interstitial tissue. Leydig cells within the interstitial tissue are responsible for producing androgens to sustain male characteristics and spermatogenesis, whereas seminiferous tubules are the site for production of spermatozoa. The male germline in the seminiferous tubules of the adult testis includes spermatogonia, spermatocytes, and spermatids. Sertoli cells provide various necessary factors for the germline cell types and support the proliferation and differentiation of immature undifferentiated germ cells to mature spermatozoa (Griswold 1993).

Sertoli cells are the nurse cells that form a simple columnar epithelium attached to the basal lamina of the tubules. Sertoli cells have many extended processes that wrap around germ cells and protrude into the lumen. Sertoli cells have several responsibilities in the testis, most notably to support and regulate the developing germ cells. They are also involved in apoptosis of germ cells and phagocytosis of excess cellular material (Barone *et al.* 2004). There is a network of tight junctions between the Sertoli cells which plays a critical role in the formation of the blood-testis barrier, and in the separation of the seminiferous epithelium into the basal and adluminal compartments (Pelletier and Byers

1992). These tight junctions provide a barrier preventing entry of immunologic cells into tubules as they may otherwise perceive haploid germ cells as abnormal cells. This is achieved by transporting the early meiotic spermatocytes from the basal to the adluminal compartment. Tight junctions, however, allow the passage of nutrients to reach more advanced germ cells. There is a special microenvironment, the stem cell niche, formed at the basal compartment where Sertoli cells support and regulate the self-renewal and differentiation of spermatogonial stem cells (SSCs) (de Rooij 2009).

2.2 Development of the male germline and seminiferous epithelium in the pig, sheep and bull

2.2.1 Embryonic and fetal development

Primordial germ cells (PGCs) are present at the earliest stage of the germline and originate from the epiblast cells at the caudal end of the primitive streak in mammals. In mice, PGCs proliferate as they migrate to the hindgut endoderm and then to the genital ridge (Anderson *et al.* 2000; Merchant-Larios and Moreno-Mendoza, 2001). Cells of the coelomic epithelium proliferate and enter through the epithelium of the genital ridge to enclose the PGCs (Karl and Capel 1998; Sharpe 2006). In sheep, PGCs begin to arrive at the genital ridge at ~24 days post-coitum (dpc), migration peaks ~31 dpc, and ceases at 41-45 dpc, while the primary gonad begins to form the testis at ~29 dpc (Zamboni and Upadhyay 1982).

In mice, the expression of a transcription factor on the Y chromosome called the sex determining region Y (SRY) initiates testicular differentiation. This factor is essential

for testicular differentiation and also affects the differentiation of glands and ducts (Albrecht and Eicher 2001; Lovell-Badge *et al.* 2002). A putative meiosis inhibitory factor released from the Sertoli cells blocks the entry of PGCs into meiosis. The expression of the SRY in the Sertoli cells of male sheep embryos activates testicular differentiation beginning at 34-35 dpc (Zamboni and Upadhyay 1982). The length of the seminiferous cords increases at 41-42 dpc to radiate to the outer half of the testis. PGCs in the center of the seminiferous cords are now called gonocytes (Sapsford 1962; Zamboni and Upadhyay 1982) which are arrested in the G0 phase of the cell cycle, starting at 14.5 dpc in mice, and remain mitotically-inactive until after birth (de Rooij and Russell 2000).

2.2.2 Postnatal development

Gonocytes migrate to the basement membrane of the seminiferous tubules, proliferate and form the primary population of SSCs in the prepubertal testis. Breed, diet and season affect the chronology of prepubertal development (Hafez 1987). As stem cells, SSCs have the ability to self-replicate (to maintain their population) and differentiate into spermatogonia. Spermatogonia undergo mitotic divisions in the testis. The number of divisions; however, differs among species, but usually ranges between 4 and 12.

After mitotic proliferation, spermatogonia undergo meiosis and give rise to primary spermatocytes. When meiosis is complete, secondary spermatocytes differentiate into round spermatids, followed by extensive morphological changes to become elongated spermatids and finally spermatozoa (de Rooij and Russell 2000; Steger and Wrobel 1994). While spermatogonia begin to proliferate and differentiate, Sertoli cells start to

secret a fluid which forms the tubular lumen, transforming the seminiferous cord into a tubule (Sapsford 1962). In mice, it has been shown that the efficiency of the first wave of spermatogenesis is less than that in adult testis, because A-spermatogonia skip several steps (de Rooij and Russell 2000). At the end of the postnatal development, the seminiferous tubules appear morphologically similar to those of adults (Sapsford 1962; Steger and Wrobel 1994).

2.2.3 Spermatogenesis

Spermatogenesis is the process of proliferation and differentiation of male germ cells to produce fertile spermatozoa. Spermatogenesis occurs in the seminiferous epithelium and consists of three phases. In the proliferative phase, a SSC starts to divide while others remain inactive as reserve. After more mitotic divisions, the daughters become A-spermatogonia which differentiate into A1-spermatogonia. Several divisions occur, dependent on the species, occur to produce more A1-spermatogonia, followed by intermediate (I)-spermatogonia and ending with in B-spermatogonia. B-spermatogonia then divide to form preleptotene spermatocytes. The diploid primary spermatocytes pass through the Sertoli cells' tight junctions to develop in the adluminal compartment and away from the body's immune system to undergo meiosis. (de Rooij and Russell 2000).

Primary spermatocytes develop into haploid spermatids through meiosis. Preleptotene spermatocytes become leptotene, zygotene and pachytene primary spermatocytes after DNA synthesis. The first meiotic division results in the formation of 2 secondary spermatocytes which undergo the second meiotic division fairly quickly to form 4 haploid round spermatids. The next step is spermiogenesis when the round spermatids

give rise to elongated spermatids. There are several stages in spermiogenesis including acrosome appearance, nucleus condensation, cell elongation, flagellum formation and finally cytoplasmic resorption and spermiation (Russell *et al.* 1990; de Rooij and Russell 2000).

At a given section of the seminiferous epithelium, depending on the species, there are 4-5 generations of germ cells at different stages of development. These stages appear sequentially throughout the seminiferous tubules with a helical or segmened arrangement. In the segmented arrangement, a wave-like release of spermatozoa occurs over time (Russell *et al.* 1990).

2.3 Spermatogonial stem cells (SSCs)

Production of spermatozoa during adulthood is sustained by SSCs which gradually self-renew and differentiate. The total number of type A_s spermatogonia (rare "single" spermatogonia thought to have stem cell potential) is ~35,000 per testis in the mouse and about 10 times more in the rat testis which is about 10 times heavier in weight (de Rooij and Russell 2000). The study of SSC biology and the factors regulating their self-renewal or differentiation is difficult because of their low number and difficulties in isolating and identifying them (de Rooij and Russell 2000). Although the morphological characteristics and location within the tubule may help distinguish SSCs *in situ*, morphology cannot be used for identification of SSCs after single cell preparation from the testis or cell culture (de Rooij and Russell 2000).

On the other hand, SSCs are unique among adult stem cells in the body because they are able to transmit genetic materials between generations. Additionally, SSCs possess plasticity allowing them to spontaneously reprogram to become embryonic stem (ES)-like pluirpotent cells even without the influence of exogenous genes. As indicated above, SSCs reside in the putative stem cell niche, formed and influenced by Sertoli cells (de Rooij 2009). Sertoli cells can exert their influence on SSCs by producing growth factors (e.g., FGF2, GNDF, activin A, and BMP4) to stimulate differentiation or self-renewal. Differentiation mostly happens outside the niche and self-renewal inside the niche. In rodents, A1 to A4, I- and B-spermatogonia are the differentiated spermatogonia. In humans, little information is available but it is proved that A-spermatogonia are divided into dark and pale types. The dark A-spermatogonia are the reserve population of stem cells while the pale are the renewing spermatogonia (Dym *et al.* 2009; de Rooij 2009; Brevini *et al.* 2008).

2.4 Spermatogonial stem cell transplantation

Germ (spermatogonial) cell transplantation is a relatively new reproductive technology in which SSCs from a donor are microinjected into the seminiferous tubules of the recipient testis. The first successful report of germ cell transplantation was published 16 years ago (Brinster and Zimmermann 1994; Brinster and Avarbock 1994). Since then, this technology has played an important role in the study, manipulation, and preservation of male fertility and spermatogenesis in rodents (Sofikitis *et al.* 2003; Dobrinski 2005). To increase the efficiency of germ cell transplantation, endogenous germ cells in the recipient testis have to be absent or destroyed to allow colonization by SSCs and

initiation of spermatogenesis. Later, it was demonstrated that rat SSCs can colonise mouse tubules and generate donor-derived rat spermatozoa in the mouse testes (Clouthier *et al.* 1996). However, SSCs from farm animals and primates may colonise the mouse testis but cannot undergo full spermatogenesis, likely as a result of the phylogenetic distance from mice (Dobrinski *et al.* 2000; Nagano *et al.* 2001). Since then, SSC transplantation technique has been adapted for use in large animals including pigs, goats, and bulls (Honaramooz *et al.* 2002; 2003; Izadyar *et al.* 2003). It has been demonstrated that goat germ cells transplanted into goat recipient testes can even establish long term donor-derived spermatogenesis where the resultant spermatozoa can be obtained from the recipient goat ejaculate (Honaramooz *et al.* 2003a; 2003b).

Germ cells are able to colonise the recipient testis even after a few weeks in culture (Nagano *et al.* 1998; Jeong *et al.* 2003). Oatley and colleagues (2004) manipulated testis tissue *in vitro* and showed that SSCs continued to proliferate in culture. One of the most important applications of this technique is the ability to confirm the presence of SSCs in a given population of testis cells from any species in the recipient mouse testis (Fredericks *et al.* 2004; Nagano *et al.* 2003; McLean *et al.* 2003). However, one of the important problems with SSC xenotransplantation is that it is relatively inefficient and cannot be applied using non-rodent donor species to obtain full spermatogenesis.

2.5 Testis tissue xenografting (TTX)

At the first glance, the testis tissue does not seem to be an ideal tissue to be used for grafting because of its complex histology and the architecture of its vascular and duct systems. However, grafting of the testis tissue has been performed since 1920s and more

recently, the availability of different lines of immunodeficient mice has allowed testis tissue and other tissues to be xenografted. Testis tissue xenografting (TTX) has proven to be a valuable and relatively easier technique than spermatogonial stem cell transplantation for the study of spermatogenesis and the male germline (Paris *et al.* 2004; Dobrinski 2005).

2.5.1 History of testis tissue xenografting

Moore (1924) grafted a newborn rat testis into the scrotum of a castrated male rat and described the presence of seminiferous tubules containing spermatozoa at 6 mo post-grafting. After the successful intra-ocular transplantation of the uterine endometrium (Markee 1932), Turner (1938) tried the same site for homologous transplantation of newborn rat testis tissue into neonatal castrated and intact male and female recipient rats of the same species. Another part of the experiment was transplanting vesicular and prostate glands to assess the endocrine function of the grafts. It was concluded that the size of the grafts is not a reliable indicator of graft survival or function. Interestingly enough, not only did the tissue survive inside the eye, but the graft survival was also 40% greater than the grafts placed at the other sites. These observations highlighted the importance of the transplantation site which should be at a low temperature for spermatogenesis to occur and also to provide suitable vascularisation for the graft. These experiments also demonstrated that testis grafts are able to produce bioactive testosterone and can even undergo complete spermatogenesis to produce elongated spermatids. Overall, this study offered a brilliant model to study spermatogenesis, testis development and its endocrine regulation.

In 1950, Williams used a transparent chamber technique in the ear of a 6-mo-old rabbit to perform an autologous grafting of testis tissue to study and describe the interaction between seminiferous tubules and the interstitial cells and their daily changes. This experiment demonstrated that seminiferous tubules needed interstitial cells to grow likely because of the testosterone secretion, but the interstitial cells were not dependent on the tubules to grow or maintain their function.

Deanesley (1954) reported that grafting of the prepubertal rat testis tissue to other rats was successful and that the tissue was able to grow and differentiate as homo-grafts after freezing and thawing. Chan *et al.* (1969) demonstrated that grafting of testis tissue from normal or pseudohermaphroditic male rats to the ear of normal or pseudohermaphroditic adult rats maintained the ability for spermatogenesis. This work confirmed that the hypothalamo-pituitary-gonadal axis is functional in these animal models.

Xenografting of human fetal testis tissue into the abdominal wall of adult nude mice was then applied in 1974, but did not result in spermatogenic development (Skakkebaek *et al.*). Gosden and Aubard (1996a, b) reviewed the reports on autologous and homologous testis tissue grafting up to that time-point (1996). Engraftment of fetal ovine testis tissue into the scrotum of intact mice demonstrated the differentiation of gonocytes to spermatogonia and primary spermatocytes as the most advanced cell types (Hochereau-de-Reviers and Perreau 1997).

Prior to the 21st century, testis tissue grafting was largely restricted to homologous and autologous grafts. A main reason that limited the potential growth of grafted tissue was the lack of widespread use of immunodeficient animals. Honaramooz *et al.* (2002), for

the first time, reported the successful application of immunodeficient nude mice as recipients for cross-species testis tissue xenografting. Immunodeficient nude mice are unable to mount cellular immune reaction against the xenografts, because they lack the thymus. In their study, Honaramooz *et al.* (2002) grafted small (~1 mm^3) fragments of testis tissue from immature mice, pigs and goats (1-2 d, 1 wk and 4 wk of age, respectively) into the back skin of castrated immunodeficient nude mice. The graft site provided an easy access for grafting and observing the graft growth. In addition, the temperature of the grafting site in the mouse (~36.5°C) was almost the same as that in the scrotum. Interestingly, not only did the testis fragments increased in size (up to 100×), but they also were able to differentiate and undergo complete spermatogenesis. The timing of somatic and germ cell differentiation in the homologous mouse grafts were almost the same as that of intact testes. However, in mouse-to-mouse grafts at 2 mo post-grafting, many of the seminiferous tubules were dilated which caused a reduction in spermatozoa production and a premature sloughing of germ cells. There appeared to be pressure necrosis of the seminiferous epithelium because of high volume of fluid secretion by Sertoli cells and lack of a duct system inside the grafted tissue to drain the tubular fluid. The timing of development and differentiation of the testis tissue, and efficiency of spermatozoa production in the grafts from immature pigs and goats were similar to the intact testis, but dilation of tubules was rarely seen. Interestingly, elongated spermatids appeared earlier in pig testis xenografts than in age-matched pig testes. This observation became a motivation for contemplating potential applications aimed at decreasing the generation interval and increasing genetic improvement. In the same study, the possibility of cryopreserving immature testis tissue prior to grafting was also demonstrated to maintain the ability to produce spermatozoa. The retrieved murine,

porcine and caprine spermatozoa could initiate fertilization of mouse oocytes after intracytoplasmic spermatozoa injection (ICSI) (Honaramooz *et al.* 2002).

In the same year, spermatogenesis was also observed in testis tissue xenografts from immature hamsters and monkeys (Schlatt *et al.* 2002), Shinohara *et al.* (2002) were able to obtain offspring using spermatozoa retrieved from xenografted testis tissue. In the latter work, the immature rabbit testis tissue used for xenografting into intact recipient nude mice, was cryopreserved prior to grafting. Schlatt *et al.* (2003) reported utilization of spermatozoa obtained from mouse-to-mouse testis tissue grafts in ICSI to produce offspring. Similar results as in that study were obtained when donor mouse testis tissue was grafted into either female or male recipient mice (Ohta and Wakayama 2004).

Therefore, the recent use of immunodeficient mice has made it possible to apply TTX to study testis function, as a valuable and relatively easy technique that is applicable in a variety of species including domestic animals and primates. This technique provides easy access to the tissue in the recipient mouse, something that is not feasible in many donor species. This possibility will allow analysis of the effects of new hormone regimens, drugs or toxicants on testis function, without the use of the target species. Improvements have also been made in cryopreservation of testis tissue to maintain its post-grafting developmental potential (Zeng *et al.* 2009; Abrishami *et al.* 2010b). Therefore, grafting of fresh or preserved testis tissue is now possible, offering an invaluable tool for the conservation of fertility even from immature gonads. For instance, TTX can provide an alternative genetic rescue strategy for neonatally lethal phenotypes or to eliminate the potential risk of tumour cell transmission which may

otherwise result from transplantation of isolated germ cells back into the testis of a cancer survivor to restore his fertility (Paris and Schlatt 2007). Another important possibility that TTX can provide is the ability to preserve the genetic materials from rare or endangered animals that die before puberty (Arregui *et al.* 2008a).

2.5.2 The routine procedure for testis tissue xenografting

Donor tissue is usually obtained through aseptic removal of the testes at castration and transferred to the laboratory in ice-cold Dulbecco's phosphate buffered saline (DPBS). The testes are then washed in DPBS containing antibiotics, and after removal of the tunica albuginea, rete testis and overt connective tissue, the testis parenchyma is divided into small fragments of ~5 mg , and maintained in Dulbecco's modified Eagle's medium (DMEM) on ice until grafting into the recipients. The recipient immunodeficient mice are anesthetised, usually gonadectomised, and receive multiple transverse linear incisions (0.5-1 cm in length) into the back skin. A fragment of the donor testis tissue is placed subcutaneously near each incision and the incisions are closed with wound clips (Honaramooz *et al.* 2002; Paris and Schlatt 2007; Rodriguez-Sosa and Dobrinski 2009). Since the time taken from the immature state of the tissue to full spermatogenesis varies among donor species, the post-grafting time-points for graft analysis are chosen based on the donor. However, after TTX, acceleration of testicular development has been observed in grafted tissue from some donor species while that of other species may remain the same as the age-matched testes or even delayed. After retrieval of the xenografts, they are processed for histology and assessed in terms of development, progression of spermatogenesis, general morphology of the seminiferous tubules, and

size of tubules, as compared with the donor tissue at grafting or with age-matched testes. Developmental progression is documented by evaluating the tubular morphology and identification of the most advanced germ cell type present in the seminiferous tubules. Furthermore, since vesicular glands are androgen-dependant and regress significantly after castration in mice, their weight can used as an important indicator of bioactive testosterone released by the grafts in castrated recipients. Serum levels of gonadotropins and testosterone could also be measured in the blood taken at the time of sacrifice (Honaramooz *et al.* 2002; 2004; Rathi *et al.* 2005; 2006; Schmidt *et al.* 2006a).

2.5.3 Testis tissue xenografting from different donor species

2.5.3.1 Porcine testis tissue xenografting

Completion of spermatogenesis following TTX from neonatal piglets into recipient mice was first reported by Honaramooz and colleagues (2002). Timing of testicular maturation in testis xenografts appeared slightly accelerated - by a few weeks - compared with the age-matched control pigs. Although some irregular formation of seminiferous tubules was observed in the xenografts, the spermatozoa production (per gram tissue) was quantitatively similar to *in situ* testes. The spermatozoa retrieved from these porcine testis xenografts were shown to be fertilization competent using ICSI in a mouse oocyte assay (Honaramooz *et al.* 2002). Later, the *in vitro* development of embryos after ICSI using porcine xenogeneic spermatozoa injected into porcine oocytes was shown to be comparable to that of testicular spermatozoa (Honaramooz *et al.* 2008). Very recently, the development of porcine embryos resulting from xenogeneic spermatozoa to term and birth of live piglets was also confirmed (Nakai *et al.* 2010). In

the latter study, spermatozoa recovered from porcine testis tissue xenografts were injected into *in vitro* matured porcine oocytes and the embryos were transferred into estrus-synchronised recipients where 2 of 23 recipient gilts gave birth to 6 piglets. Therefore, the ability of the spermatozoa from ectopic testis tissue xenograft to result in viable offspring has now been shown in a large mammal model.

Zeng and colleagues (2006) demonstrated that the length of the spermatogenic cycle is conserved in porcine and ovine testis xenografts; therefore, this timing is inherent to the tissue. They also proposed that (exogenous) gonadotropin stimuli could decrease the time required for maturation of testis xenografts, without changing the timing of spermatogenic cycles (Zeng *et al.* 2006). The same groups of researchers later showed that the global gene expression in porcine testis xenografts is comparable to that of testis tissue *in situ* (Zeng *et al.* 2007). These results supported the notion that TTX has great potential as a model system in replicating the *in situ* tissue for the study and manipulation of mammalian spermatogenesis and its regulation.

2.5.3.2 Ovine and caprine testis tissue xenografting

Ectopic TTX from immature sheep and goat donors into recipient mice also resulted in complete spermatogenesis in the grafts (Honaramooz *et al.* 2002; Arregui *et al.* 2008a). Goat testis tissue xenografts underwent testicular maturation and completed spermatogenesis at a rate similar to that of the testis *in situ*, whereas the timing of testicular maturation was relatively accelerated in the ovine testis tissue xenografts in one study (Dobrinski *et al.* 2003) but not in others (Zeng *et al.* 2006; Arregui *et al.* 2008a). Interestingly, the quantity of spermatozoa production (per gram tissue) in goat

testis tissue xenografts was comparable to that of testes *in situ* and the resultant spermatozoa were fertile after ICSI in a mouse oocyte assay (Honaramooz *et al.* 2002).

Similar to using domestic donor kittens as a model for TTX from endangered felids (Snedaker *et al.* 2004), immature donor rams have been suggested to provide a suitable model for the assessment of TTX from endangered bovids (Paris and Schlatt 2007; Arregui *et al.* 2008a). It is believed that more than 80% of the endangered bovid species are closer to the subfamily Caprinae than Bovinae (Herna´ndez Ferna´ndez and Vrba 2005) and according to the red list of threatened species, sheep and goats could be better models for 140 species of bovidae that are under some degree of threat (IUCN 2010, www.iucnredlist.org).

2.5.3.3 Bovine testis tissue xenografting

Ectopic xenografting of bovine testis tissue into recipient mice resulted in moderate graft recovery rates and delayed, yet eventual completion of spermatogenesis. Although the donor tissue from bull calves of different tested ages completed testicular maturation and spermatogenesis, the grafts from older donors showed slow progression of spermatogenesis over time (Rathi *et al.* 2005; Schmidt *et al.* 2006a; Huang *et al.* 2008). This finding confirmed the importance of donor age on the outcome of TTX (Oatley *et al.* 2005; Schmidt *et al.* 2006a). There are conflicting reports on the timing of testicular maturation in bovine testis tissue xenografts, with one showing a similar rate to the *in situ* testis (Oatley *et al.* 2004), while another study from the same group showed acceleration of this timing (Schmidt *et al.* 2006a). Rathi and colleagues (2005) using TTX from neonatal donor bull calf demonstrated a rapid rate of post-grafting increase in

the seminiferous tubule lumen formation, pre-meiotic differentiation and pachytene spermatocytes production, but a slow post-meiotic differentiation of germ cells. They also observed moderate over-dilation of some of the seminiferous tubules (Rathi *et al.* 2005), although it was not as severe as that seen in mouse testis tissue allografts (Honaramooz *et al.* 2002). Furthermore, presence of more advanced germ cells in the donor tissue at the time of grafting has been correlated with poor post-grafting testicular development and differentiation, whereas the abundance of undifferentiated germ cells at the time of grafting results in better graft development (Huang *et al.* 2008).

Researchers working on bovine TTX have reported facing challenges including the loss of germ cells and low efficiency of spermatogenesis in the grafts from this species as compared with other domestic species (Oatley *et al.* 2004; Rathi *et al.* 2005; Schmidt *et al.* 2006a). Explanations offered for this observation include an initial pre-meiotic arrest or ineffective repopulation which could lead to the low number of germ cells in the grafts. Schmidt and colleagues (2006b) demonstrated that treating the bovine testis tissue fragments with vascular endothelial growth factor (VEGF) at the time of grafting (to help neo-angiogenesis) could increase graft weight and the percentage of seminiferous tubules containing elongated spermatids. Therefore, this latter study demonstrated for the first time that testis tissue could be manipulated prior to xenografting to provide better results in term of spermatogenesis, cell differentiation and producing more advanced germ cell types. In a subsequent study, the same group suggested that genetic analysis of the tissue at the time of grafting as well as during the post-grafting period can provide important information about the pattern of gene expression in the xenografted testis tissue during development, and that manipulation of

xenografts microenvironment could potentially result in improving the efficiency of spermatozoa production (Schmidt *et al.* 2007).

2.5.4 Factors affecting the outcomes of ectopic testis tissue xenografting

2.5.4.1 Effects of donor factors

2.5.4.1.1 Donor species

Unlike germ cell transplantation, TTX from species that are phylogenetically distant from rodents has been successful. This method has been applied using different donor species from rodents to farm animals and primates and in all instances, the grafts appeared to be responsive to the gonadotropins provided by the recipient mice in a way that was sufficient for the tissue to complete maturation and spermatogenesis (Honaramooz *et al.* 2002). The only exception was when the common marmoset (*Callithrix jacchus*) donor testes were used for TTX and no post-meiotic development occurred in the grafted tissue (Honaramooz *et al.* 2003c), although complete spermatogenesis was observed after transplant of testis tissue from the rhesus monkey (*Macaca mulatta*) (Honaramooz *et al.* 2003c). A deletion in exon 10 of the LH-receptor gene was responsible for making the marmoset testis non-responsive to LH (Honaramooz *et al.* 2003c), including LH from the recipient mouse. This finding highlighted the importance of LH for the post-meiotic development of male germ cells as the reason for the poor spermatogenesis and androgen production in the marmoset testis tissue xenografts (Schlatt *et al.* 2003; Wistuba *et al.* 2004).

Exposure of the immature donor testis tissue to the adult-fashion mode of gonadotropin release in the recipient mouse results in maturation of the permissive immature testis tissue xenografts and initiation of spermatogenesis earlier than *in situ* testes (Honaramooz *et al.* 2002; 2004). The efficiency of spermatogenesis based on the number of spermatozoa produced per gram of tissue was comparable to that of age-matched intact testes in xenografts from immature pigs, goats, sheep and rhesus monkeys (Honaramooz *et al.* 2002; 2004; 2008), whereas, this efficiency was lower in xenografts from bull calves, cats, horses and dogs (Oatley *et al.* 2004; 2005; Rathi *et al.* 2005; 2006; Schmidt *et al.* 2006a; Snedaker *et al.* 2006; Abrishami *et al.* 2010a). Therefore, the efficiency of complete spermatogenesis in testis tissue xenografts may be dependent on the donor species.

2.5.4.1.2 Donor age

Donor age and developmental status of the donor testis tissue at the time of grafting is another important factor affecting the efficiency and outcome of TTX. Several studies have shown that TTX from mature donor testis tissue does not provide a suitable model for this technique (Schlatt *et al.* 2002; Geens *et al.* 2006; Kim *et al.* 2007; Arregui *et al.* 2008b; Abrishami *et al.* 2010a). The spermatozoa observed following TTX from mature human and mouse donor testis tissue were limited in number and were thought to have been as a result of development of pre-existing germ cells and not from newly-developed waves of spermatogenesis (Schlatt *et al.* 2002; 2006; Geens *et al.* 2006; Abrishami *et al.* 2010). There also seemed to be variations in timing of tubular degeneration after TTX from mature donors, since for example, this degeneration

happened earlier in the post-grafting period, in mature pig and goat donor tissues than in those from other mature donor species (Arregui *et al.* 2008b). The extent of this degeneration appeared to be dependent on the level of sexual maturity of the donor, since the degree of seminiferous tubule degeneration in the grafts increased with maturity of the donor tissue (Arregui *et al.* 2008b; Abrishami *et al.* 2010a). The inability of the adult testis tissue to thrive after xenografting has been attributed to the complexity of the tissue, its sensitivity to ischemia, and its inability for neo-angiogenesis, compared with the immature testis tissue (Honaramooz *et al.* 2002; Schlatt *et al.* 2006). Another reason that could explain the lower survival rate and higher degeneration of adult testis xenografts is the inability of adult Sertoli cells to proliferate and maintain the complex structure of the testis tissue (Schlatt *et al.* 2002; Meachem *et al.* 2005; Arregui *et al.* 2008a,b).

Although neonatal and prepubertal animals have been generally shown to provide the best donors for TTX, there still seemed to be some differences in the outcomes of experiments using immature testis tissue donors, depending on their age and the status of testicular maturation. In a study of bovine TTX, it was demonstrated that at the time of graft retrieval, the grafts from 8-wk-old donors had more seminiferous tubules containing elongated spermatids than those of 4- or 12- to 16-wk-old donor tissues (Oatley *et al.* 2005). Although both 4- and 8-wk-old testis xenografts contained gonocytes or spermatogonia at the time of grafting, it was proposed that the 8-wk-old donors were at a relatively higher developmental stage than those of the 4-wk-old donors. The same group of researchers later reported that testis tissue xenografts from 1- and 8-wk-old donor bull calves produced higher percentages of complete

spermatogenesis than 4-wk-old donors; however, extending the post-grafting time made a significant improvement on the extent of spermatogenesis in xenografts from 4-wk-old donors (Schmidt *et al.* 2006a).

Meiotic and post-meiotic germ cells were also reported to be lost at 4 mo post-grafting in equine xenografted testis tissue that had reached meiosis at the time of grafting (Rathi *et al.* 2006). Abrishami *et al.* (2010a) using TTX from dogs of a wide range of ages concluded that immature donors (<3 mo old) were the most promising donors, adult tissue donors were not suitable for TTX and among prepubertal tissue donors the results were variable in terms of graft recovery, growth, and development of the xenografts over time. It has been suggested that during the grafting procedures and immediately afterward, all testis tissue fragments undergo some degree of damage due to hypoxia and ischemia; however, this seemed to be a problem particularly for differentiated germ cells since the immature tissue is better able to bounce back from the damage (Schlatt *et al.* 2002; Rathi *et al.* 2006). Therefore, testis tissue xenografts from newborn and prepubertal donors are overall better in survival rates, cell differentiation and support of spermatogenesis than those of the donor tissues with pre-existing spermatogenesis at the time of grafting.

2.5.4.2 Recipient factors

2.5.4.2.1 Transplantation site

Turner (1938) demonstrated that the anterior chamber of the recipient rat's eye was a better site for homologous grafting of testis tissue as compared with the subcutaneous,

intra-peritoneal, scrotal, or intra-muscular sites. He cited the lower temperature and more vascularisation in the eye as likely reasons for these observations. Low temperature and high potential for vascularisation are still considered to be key factors for making a site suitable for TTX. In the first report of successful testicular maturation and completion of spermatogenesis following TTX from diverse species into mice (Honaramooz *et al.* 2002), the tissue fragments were sutured subcutaneously under the back skin of immunodeficient nude mice. Since the nude mice do not have a fur coat, one might assume that xenografts developing under the skin are exposed to a relatively lower temperature than those in mice with fur. However, Snedaker *et al.* (2004) suggested that there is no difference in spermatogenic efficiency between nude and SCID mice (which have complete fur). Shinohara *et al.* (2002) also reported successful xenografting of neonatal testis tissue under the tunica albuginea of the recipient mouse testes. However, most of the other studies utilizing TTX have shown that the subcutaneous space under the back skin of recipient mice as the ideal site for grafting testis tissue fragments, since it provides a low temperature, is highly vascularised, and easily accessible for growth and development of the xenografts.

2.5.4.2.2 Hormonal status of the recipients

Following the procedures described by the first report of TTX from diverse species (Honaramooz *et al.* 2002), almost all studies on this subject have used castrated male mice as recipients. Castration of the recipient mouse prior to TTX was believed to prevent complications between endogenous products of recipient's testes and those of the newly grafted testis tissue. The mouse accessory sex glands, particularly the

vesicular glands, are androgen-dependent and will regress to about 10% of their normal weight (100-150 mg) within a few weeks after castration (Gosden and Aubard 1996b). This process is reversible and therefore, measuring the weight of the vesicular glands in recipient mice castrated prior to TTX will provide an androgen bioassay to evaluate the androgen producing potential of the grafted testis tissue (Honaramooz *et al.* 2002; Schlatt *et al.* 2003). Furthermore, castration of the recipient mouse causes serum concentrations of gonadotropins to increase and thereby provide a gonadotropic stimulus for the newly-grafted testis tissue (Honaramooz *et al.* 2002; Schlatt *et al.* 2003). This additional gonadotropin release may provide an instant support for the proliferation of Sertoli cells, and cause other maturational changes in the grafted immature testis tissue, before xenografts are capable of producing enough androgens to establish a hormonal feedback axis with the hypothalamus and pituitary gland of recipient. Original observations by Turner (1938) also indicated that homologous testis tissue grafts developed better and had less degeneration when recipient rats were castrated. Rathi and colleagues (2006) also concluded that because testis tissue xenografts are sensitive to competition from the recipient's gonads, they could not grow and mature in intact male recipients. However, both intact and castrated recipients were reported to support completion of spermatogenesis in rabbit testis tissue xenografts (Shinohara *et al.* 2002). Therefore, the necessity of castration for success of TTX is still controversial.

2.5.4.2.3 Recipient mouse strain

Genetic manipulation of laboratory animals has provided a variety of valuable immunodeficient recipient models available for grafting studies. Some of the more

commonly used strains are nude (nu/nu), severe combined immunodeficient (SCID), and NOD/Shi-SCID IL-2Rγc^{null} (NOG) mice.

The nude mouse is so-named because it has little or no hair. Although this feature is useful in subcutaneous grafting studies where the progress in size of the grafts can be visible over time, more importantly these mice lack the thymus. These traits result when the mouse is homozygous for a recessive mutant gene designated *Foxn1*. Due to absence of the thymus, nude mice cannot generate mature T lymphocytes and therefore are unable to mount complete cell-mediated immune responses, preventing them from rejecting allografts as well as xenografts. However, nude mice have an almost intact humoral immunity through B lymphocytes, allowing them to mount an antibody immune response against circulating antigens (Pelleitier and Montplaisir 1975; Kubota *et al.* 1993). Although they have a complete fur coat, SCID mice lack both T and B cells (Mueller and Reisfeld 1991; Kubota *et al.* 1993). A mutation of a protein kinase (*Prkdc*: protein kinase, DNA activated, catalytic polypeptide) is the cause of the phenotype seen in SCID mice (Bosma *et al.* 1983; Kirchgessner *et al.* 1995). In a TTX study where repeated administration of exogenous hormones for an extended period of time was necessary and the researchers were concerned about possible antibody response in nude mice, SCID mice were used as the recipients (Honaramooz *et al.* 2004). The NOG mice have multiple mutations which in essence are derived from three immunodeficient strains of their origin, including the lack of macrophage function, T and B cells, as well as NK cells. These immunodeficiencies make these mice extremely vulnerable to infections and therefore challenging to maintain for long-term.

To date, a number of indirect comparisons of the outcomes of TTX have been made between nude and SCID recipient mice, but no conclusive differences were found. Clouthier and colleagues (1996) concluded that nude mice were better recipients for germ cell transplantation than SCID mice. Honaramooz *et al.* (2004; 2008) used SCID mice as recipient for TTX using both immature rhesus monkeys and piglets and reported obtaining fertilisation-competent spermatozoa from the grafts. Rathi *et al.* (2006) concluded that the development or maturation of equine testis xenografts did not differ between nude and SCID recipients. It has been suggested that a higher severity of immunodeficiency in recipient mice may provide a more suitable host environment for the development of xenografts (Ito *et al.* 2002). Watanabe *et al.* (2009) used three different strains of recipients (nude, SCID and NOG mice) to study ectopic porcine spermatogenesis by TTX and germ cell injection methods. They reported that all recipients supported the same extent of spermatogenesis and that SCID mice were the best model for receiving germ cell injections. They also concluded that the severity of immunodeficiency is not a determining factor for the extent of spermatogenesis since they did not observe any better results from NOG mice. However, firm conclusions were difficult because of the small animal sample sizes used in the latter study.

2.5.5 Application of testis tissue xenografting in endangered species

Based on the Species Survival Commission (2008) of the International Union for Conservation of Nature (IUCN, www.iucnredlist.org), 5,966 of 26,604 vertebrate species assessed (22%) are currently threatened. There are 1,141 mammalian species on the IUCN's Red List of Threatened Species (from vulnerable to extinct), 72 of which are

in Canada. A closer look at this statistical analysis shows that one in three amphibian species, one in eight bird species, and one in five mammal species is threatened by extinction. Preservation of the habitant has been suggested as the first priority for reducing the speed of decline of animal species (Hanks 2001), however, this attempt is not enough and so far such efforts have not been successful. Breeding and spreading the species *ex situ* may be applicable for some species (Bainbridge and Jabbour 1998). Preserving immature gametes and pluripotent/germline stem cells may also provide a novel and promising solution. Given the advances in cryopreservation of testicular and ovarian tissues, reproductive tissue banking may also be considered as an option to preserve germlines from endangered or rare species.

When a member of an endangered species or valuable wild animal dies, its potential contribution to the genetic pool is permanently lost. In adult males, spermatozoa can be collected and cryopreserved before or even shortly after death, and the spermatozoa can be used for assisted reproductive technologies (Roldan *et al.* 2006). However, preservation of spermatozoa is not an option in sexually immature males and *in vitro* culture of immature testis cells has not been completely successful. Therefore, ectopic xenografting of testis tissue may be a unique solution for the problem of rescuing the genetic material of males that die prematurely (Pukazhenti *et al.* 2006; Zeng *et al.* 2006). TTX is the only technique that can produce spermatozoa from immature testis fragments. Obviously there is no single solution for the problem of endangered species but TTX has already been applied to study and manipulate the male germline material of several donor species (Paris and Schlatt 2007). Mouse and pig offspring have been generated following ICSI using spermatozoa retrieved from testis tissue xenografts from

neonatal donors (Schlatt *et al.* 2003; Nakai *et al.* 2010). There are also reports of producing offspring from spermatozoa developed in rabbit testis tissue xenografts that cryopreserved prior to grafting (Shinohara *et al.* 2002).

To date, sheep and cats have been used as donor models for the study of TTX from closely related endangered species (Snedaker *et al.* 2004; Arregui *et al.* 2008a). Ectopic TTX was also applied to salvage the genetic potential of two cloned Javan banteng that died shortly after birth (Honaramooz *et al.* 2005).

Currently, there are 140 species of bovidae and 55 species of cervidae family under some degree of threat (IUCN 2010, www.iucnredlist.org) which could benefit from the TTX technique. To expand this technique into more species and to explore the possibility of its application in rare or endangered ungulates, we first need to apply this technique in a number of specialised hoofstock, the result of which may be applicable in closely-related wild species. More specifically, farmed bison and white-tailed deer could be excellent model species for endangered bovid and ungulates. The outcomes of TTX from these species could be extended into other bovidae and cervidae species because of the similarities in reproductive biology of bison and white-tailed deer and those of nondomestic hoofstock.

II. OBJECTIVES AND HYPOTHESES

Hypothesis 1: Xenografting of testis tissue from piglet donors into different recipient types will result in differing degrees of grafting efficiency.

***Objective 1*:** To determine which combination of recipient factors (SCID *vs*. nude, male *vs.* female, and gonadectomised *vs.* intact mice) will provide the best efficiency for TTX.

Hypothesis 2: Xenografting of different numbers of testis tissue fragments from piglet donors into castrated male nude mice will result in different graft recovery rates and maturational status of the grafts.

Objective 2: To determine the number of testis tissue fragments grafted per mouse (2, 4, 8 or 16) that will be most efficient for TTX.

Hypothesis 3: Xenografting of testis tissue from specialised livestock into recipient mice will result in graft recovery, testicular maturation and initiation of spermatogenesis in the testis xenografts.

Objective 3: To evaluate the long-term outcome of TTX (graft recovery rate, timing of testis maturation and spermatogenesis) from specialised livestock donors into recipient mice.

CHAPTER 3: THE EFFECTS OF RECIPIENT MOUSE STRAIN, GENDER AND GONADAL STATUS ON THE OUTCOME OF TESTIS TISSUE XENOGRAFTING[1]

3.1 Abstract

The objective of this study was to examine recipient factors that may affect the outcome of testis tissue xenografting (TTX). Eight small fragments of testis tissue from newborn piglets were grafted under the back skin of immunodeficient mice of different strains (SCID *vs.* nude), gender (male *vs.* female), and gonadal status (intact *vs.* gonadectomised), using a factorial design (8 groups, n = 7 mice/group). Recipient mice were sacrificed after 8 mo to compare the gross and histological attributes of the recovered grafts. Gonadectomy of male or female recipients did not affect any of the measured outcomes of TTX; therefore the data were pooled. Overall, male SCID mice tended to show higher gross and histological development of grafts. The graft recovery rate was lowest in the female nude mice (75%); recovery rate was 95-100% for the other groups ($P < 0.05$). The grafts from male SCID mice were, on average, the largest and had the highest percentage of spermatozoa-containing seminiferous tubules among groups ($P < 0.05$). Male SCID mice are a suitable recipient for TTX, and they do not need to be castrated for optimal results.

[1] Manuscript has been accepted for publication in *Reproduction, Fertility and Development*.

3.2 Introduction

Spermatogenesis is a highly organised cyclic process of germ cell proliferation and differentiation that continuously produces spermatozoa throughout adulthood. The *in situ* study of testicular maturation and regulation of spermatogenesis in humans and large animals is difficult and requires suitable models (Russell *et al.* 1990). To date, *in vitro* models of spermatogenesis have not been able to fully replicate the testicular environment (Gassei and Schlatt 2007; Huleihel *et al.* 2007). Testis tissue xenografting (TTX), in which small fragments of testis tissue from a donor are grafted under the skin of recipient immunodeficient mice, has provided an *in vivo* model for testis function (Honaramooz *et al.* 2002). This technique has been applied to the study of male gonadal development in a range of donor species (reviewed by Rodriguez-Sosa and Dobrinski 2009). In this model, the grafted tissue from neonatal donors underwent testicular maturation, and progression of spermatogenesis up to the stage of spermatozoa production (Honaramooz *et al.* 2002; 2004; Schlatt *et al.* 2002; 2003; Snedaker *et al.* 2004; Rathi *et al.* 2005). The spermatozoa recovered from testis xenografts have been used for intra-cytoplasmic spermatozoa injection (ICSI), resulting the production of offspring from newborn donor animals (Honaramooz *et al.* 2002; 2008; Schlatt *et al.* 2002; Ohta and Wakayama 2005; Jahnukainen *et al.* 2006; Nakai *et al.* 2010). By maintaining the structural integrity and cell associations within the donor tissue, TTX has provided a functional environment for *ex situ* testicular maturation and spermatogenesis.

The extent of spermatogenic development in recovered testis grafts has varied widely among reports (reviewed by Dobrinski 2005). Differences due to donor species or developmental stage of testis tissue have been documented, with grafts originating from immature donors generally providing superior results when compared with those of mature donors (Abrishami *et al.* 2010a, Arregui *et al.* 2008b). Other sources of such variation are unknown, since even using the same donor testis tissue for xenografting into multiple mice has led to some degree of inconsistency in results (Honaramooz *et al.* 2002; Oatly *et al.* 2004; Schmidt *et al.* 2006a). There are indications that variation in recipient mouse models may be a contributing factor affecting the outcome of TTX (Ehmcke *et al.* 2008).

Nude mice, which lack T lymphocytes and cell-mediated immunity, have been used as recipients in the majority of TTX studies (reviewed by Rodriguez-Sosa and Dobrinski 2009). It has been suggested that a higher severity of immunodeficiency in recipients may provide a more suitable host environment for the development of xenografts (Ito *et al.* 2002). Severe combined immune-deficient (SCID) mice, which lack both T and B lymphocytes, have also been used successfully for TTX with no obvious improvement in the outcomes (Rathi *et al.* 2005; 2006; Geens *et al.* 2006; Goossens *et al.* 2008; Watanabe *et al.* 2009).

Recipient mice are normally castrated before or at the time of grafting, causing serum concentrations of gonadotropins to increase (Schlatt *et al.* 2003). This post-castration rise in gonadotropins levels is perceived to be useful, or even necessary, to provide gonadotropic stimulation for the newly-grafted testis tissue (Honaramooz *et al.* 2002;

Dobrinski 2005; Paris and Schlatt 2007; Rodriguez-Sosa and Dobrinski 2009). Furthermore, in almost all studies involving TTX, the recipient mice were male. However, the effects of recipient mouse gender or gonadectomy on the outcome of TTX have not been systematically investigated.

The aim of this study was to determine the effects of recipient mouse strain, gender and gonadal status on the outcome of TTX from piglets.

3.3 Materials and Methods

3.3.1 Study design

Testis tissue from neonatal piglets was divided into small fragments and grafted under the skin along the back of immunodeficient mice. The effects of the recipient mouse strain (SCID *vs.* nude), gender (male *vs.* female) and gonadal status (intact *vs.* gonadectomised) on the outcome of TTX were investigated by grafting the donor testis tissue into 8 different groups of mice, using a factorial design. The recipient mice were sacrificed at 8 mo post-grafting and the xenografts were recovered for assessment of graft weight, recovery rate and histological development. Experimental procedures involving animals were approved by the University of Saskatchewan's Institutional Animal Care and Use Committee.

3.3.2 Donor testis tissue

Donor testes were obtained after aseptic castration of 1-wk-old Yorkshire-cross piglets (Camborough-22 × Line 65, PIC Canada Ltd., Winnipeg, MB, Canada) at a university-

affiliated swine facility. Testes were transferred to the laboratory within 1 h after collection in ice-cold Dulbecco's phosphate buffered saline (DPBS, Cat. No. 20-031-CV, Mediatech, Manassas, VA, USA) containing 2% w/v antibiotic/antimycotic solution (Cat. No. 30-004-CI, Mediatech). The testes were then washed 3 times with DPBS and the tunica albuginea, rete testis and overt connective tissue were removed. The testis parenchyma was then divided into small fragments of approximately 5 mg or 1 mm^3 in volume, and maintained in Dulbecco's modified Eagle's medium (DMEM, Cat. No. 10-013-CM, Mediatech) on ice until grafting into recipient mice within 2 h. Prior to grafting, representative donor testis tissue fragments were fixed in Bouin's solution overnight, washed with and kept in 70% v/v ethanol and processed for histology as a reference for graft development.

3.3.3 Recipient mice and procedures for xenografting of testis tissue

Two different strains of immunodeficient mice, nude (NCr, nu/nu, Taconic, Germantown, NY, USA) and severe combine immunodeficient (ICR-SCID, Taconic) were used. Mice were ~10 wk old at the time of grafting and maintained aseptically in groups of 3 or 4 in perplexiglass microinsulators under controlled photoperiod environment (lights on from 0600 through 1800) with sterile water and mouse chow provided *ad libitum*. For each strain, half of the male and female mice were randomly assigned to undergo castration/ovariectomy and the remaining mice were left intact. The recipient mice were categorised based on the strain, gender and gonadal status into 8 groups (n = 7 mice/group): intact male SCID, gonadectomised male SCID, intact female SCID, gonadectomised female SCID, intact male nude, gonadectomised male nude,

intact female nude, and gonadectomised female nude. In preparation for surgery, the mice were anesthetised with intra-peritoneal injection of ketamine hydrochloride (100 mg/kg; Ketalene, Bimeda-MTC, Cambridge, ON, Canada) and xylazine hydrochloride (10 mg/kg; Vet-A-Mix, Shenandoah, IA, USA).

Each mouse received 8 transverse linear incisions (~5 mm in length) into the back skin, 4 on each side of the midline. A small subcutaneous pouch was made in each incision and a testis tissue fragment was inserted and the incision was closed using wound clips (Michel Clips 7.5 mm, Miltex, York, PA, USA).

3.3.4 Gross and histological analysis

Recipient mice were anesthetised and sacrificed at 8 mo post-grafting. Visible testis tissue xenografts were dissected out and individually weighed, and the body weight of the mouse after removing the grafts was also recorded. The retrieved grafts were fixed in Bouin's solution overnight, washed with and kept in 70% ethanol solution until processing for histology. The fixed tissues were then processed, paraffin blocked and sectioned (at 6 µm thickness) at the largest diameter of the graft. The sectioned tissues were stained with haematoxylin and eosin and analysed under light microscopy equipped with a digital camera.

For histological analysis of the xenografts, a total of 16 images were captured from random areas of graft cross-sections from each mouse with at least one image per graft. The images were taken at 200× magnification using a calibrated microscope and labelled anonymously and the operator blindly analysed each image. All seminiferous tubule

cross-sections (or up to 200, if there were more) within the graft tissue images were evaluated (Image pro Express, version 6.0.0.319 for Windows XP/Professional, Media Cybernetics, Inc, MD, USA).

The evaluation included counting tubule cross-sections to calculate the tubular density (per mm^2), measuring the widest tubular diameter and examining tubular morphology and the most advanced germ cell type present in each tubule cross-section.

The endpoints for morphology of the seminiferous epithelium cross-sections and determination of the most advanced germ cell type present were: 1) spermatozoa present (including fully-detached spermatozoa within the lumen or when only the head remained attached to Sertoli cells and the fully-formed tail suspended in the lumen); 2) elongated spermatids present as the most advanced germ cells; 3) round spermatids present as the most advanced germ cells; 4) spermatocytes present as the most advanced germ cells (including all stages of primary and secondary spermatocytes); 5) spermatogonia present as the only type of germ cells (including all stages of A- and B-spermatogonia); 6) Sertoli-cell-only tubule (where none of the germ cell types were present); and 7) complete tubular degeneration or fibrosis (where all or most cells within the tubule were degenerated and or composed of fibroblasts).

3.3.5 Statistical analysis

Since the individual mouse was considered an experimental unit, data obtained from all recovered testis tissue xenografts within a mouse were averaged for the mouse. The endpoints considered in this study included the mouse body weight (g), graft recovery

rate (% of visible grafts retrieved compared with the original number of fragments grafted), total and average graft weight (mg), vesicular gland index (%), seminiferous tubule diameter (μm), tubular density (/mm^2), the percentages of tubule cross-sections with spermatozoa, elongated spermatids, round spermatids, spermatocytes, spermatogonia as the most advanced germ cell type, as well as the percentages of graft cross-sections displaying degeneration or Sertoli-cell-only.

We originally used a 3-way ANOVA for three main factors, strain (SCID *vs.* nude), gender (male *vs.* female) and gonadal status (intact *vs.* gonadectomised) using Sigmastat (Sigmastat for Windows Version 3.5, Systat Software, San Jose, CA, USA). The effect of gonadal status was not significant for any of the analysed parameters and there were no interactions between gonadal status and other factors. Therefore, gonadal status was removed as a factor, and the data were pooled and re-analysed for two main factors of strain and gender using 2-way ANOVA. Percentages were transformed (using Arcsin function) prior to analysis using ANOVA. The Pearson product-moment correlation coefficients were calculated for the mouse body weight, average or total graft weight and the above-mentioned examined parameters of testis graft development. Data are expressed as means ± SEM. $P < 0.05$ was considered significant.

3.4 Results

3.4.1 Mouse body weight, graft weight and graft recovery rate

Out of 56 recipient mice, 14 died (8 SCID and 6 nude) prior to the scheduled time of sacrifice and were excluded from data analysis. In the remaining 42 mice, there was a

tendency for body weight to differ based on gender (32 ± 0.8 *vs.* 29 ± 1.5 g, male *vs.* female, P = 0.054), but not strain (31 ± 1.7 *vs.* 30 ± 0.6 g, SCID *vs.* nude, P = 0.3) or their interactions. A total of 316 out of 336 grafts were recovered (overall 94% graft recovery). Mouse strain (98 ± 2.4% *vs.* 87 ± 2.3%, SCID *vs.* nude, P = 0.001), gender (97 ± 2.3% *vs.* 88 ± 2.4%, male *vs.* female, P = 0.004) and their interactions (P < 0.001) affected the graft recovery rate. The group of female nude mice had the lowest graft recovery rate (75%), whereas recovery rate was 95-100% for the other three groups (P < 0.05, Table 3.1).

At the time of grafting, testis tissue fragments weighed approximately 5 mg. At 8 mo post-grafting, the average graft weight had increased in all groups (P = 0.0002). Overall, the mouse strain (292 ± 27.0 *vs.* 160 ± 26.3 mg, SCID *vs.* nude, P = 0.001) and gender (348 ± 26.3 *vs.* 104 ± 27.0 mg, male *vs.* female, P < 0.001) affected the mean graft weight. The group of male SCID mice had the heaviest grafts, followed by male nude mice, female SCID mice, and female nude mice (P < 0.05, Table 3.1).

Table 3.1 Characterisation of testis tissue xenografts recovered from recipient mice at 8 mo post-grafting.

	SCID		Nude	
	Male	Female	Male	Female
Number of mice analysed*	8	12	14	8
Mouse body weight (g)	34 ± 2.0	29 ± 2.5	31 ± 0.5	28 ± 1.1
Graft recovery rate (%)	95 ± 4.7^{a}	100 ± 0.0^{a}	99 ± 0.9^{a}	75 ± 7.1^{b}
Average graft weight (mg)	423 ± 54.9^{a}	162 ± 15.2^{b}	274 ± 42.0^{b}	47 ± 15.4^{c}
Tubular diameter (µm)	155 ± 5.1^{a}	102 ± 5.5^{b}	148 ± 4.3^{a}	118 ± 9.8^{b}
Tubular density (/mm^2)	29 ± 2.3^{b}	54 ± 2.7^{a}	32 ± 1.5^{b}	36 ± 5.0^{b}
Spermatozoa (% of tubules)	41 ± 3.1^{a}	5 ± 1.1^{c}	26 ± 4.2^{b}	7 ± 4.3^{c}
Elongated spermatids (% of tubules)	15 ± 1.7^{a}	3 ± 0.9^{b}	11 ± 1.9^{a}	4 ± 1.7^{b}
Round spermatids (% of tubules)	11 ± 1.5^{a}	7 ± 1.4^{ab}	9 ± 1.8^{a}	3 ± 1.4^{b}
Spermatocytes (% of tubules)	11 ± 2.3^{c}	38 ± 3.5^{a}	9 ± 1.3^{c}	20 ± 5.3^{b}
Spermatogonia (% of tubules)	1 ± 0.5^{b}	12 ± 3.5^{b}	9 ± 3.7^{b}	24 ± 6.3^{a}
Sertoli-cell-only (% of tubules)	7 ± 1.3^{b}	26 ± 3.9^{a}	15 ± 3.2^{ab}	25 ± 7.7^{ab}
Degenerated (% of tubules)	14 ± 2.1	9 ± 2.8	20 ± 2.5	17± 5.5

Different groups of recipient mice were grafted with 8 fragments of porcine testis tissue, and at 8 mo post-grafting the xenografts were evaluated for spermatogenic development. Recipients were originally categorised based on strain, gender and gonadal status into 8 groups; however, gonadal status (gonadectomised *vs.* intact) had no effects and the data were pooled into four groups based on strain and gender.

* The number of recipient mice per combined group that survived to the time of analysis at 8 mo post-grafting (the original number was 14 mice per combined group).

**Graft recovery rate was defined as the relative number of visible xenografts retrieved, compared with the number of fragments grafted (8 fragments/mouse/group, n = 14 mice/group). The average graft weight and histological evaluations were based on the recovered grafts. The average seminiferous tubule diameter and tubular density (the number of the seminiferous tubules/mm^2) were calculated in the largest cross-section of the recovered grafts. For comparison, at the time of grafting, the donor tissue had seminiferous cords that were 47 ± 1.1 µm in diameter and at a density of 95 ± 6.1 tubules/mm^2. Data are presented as mean ± SEM. ^{abc} Values with different superscript letters within each row are significantly different ($P < 0.05$).

3.4.2 Histological evaluation of the recovered testis grafts

3.4.2.1 Tubular diameter

At the time of grafting, the neonatal donor testis tissue had seminiferous cords that were 47 ± 1.1 µm in diameter. Overall, the average diameter of seminiferous tubules in recovered grafts differed based on gender (150 ± 3.3 *vs.* 108 ± 5.3 mg, male *vs.* female, $P < 0.001$), but not strain ($P = 0.5$). Consequently, grafts from both groups of male SCID and nude recipient mice had higher diameters of seminiferous tubules than both groups of female recipients ($P < 0.05$, Table 3.1).

3.4.2.2 Tubular density

Seminiferous tubular density in the neonatal donor testis tissue prior to grafting was 95 ± 6.1 tubules/mm^2. There was an effect of strain ($P = 0.02$) and gender ($P = 0.0001$) of recipient mice and their interaction ($P = 0.002$) on tubular density (44 ± 3.3 *vs.* 33 ± 2.1 tubules/mm^2, SCID *vs.* nude, and 31 ± 1.3 *vs.* 47 ± 3.2, male *vs.* female, respectively). Cross-sections of grafts from the group of female SCID mice had the highest seminiferous tubular density, among the four groups ($P < 0.001$, Table 3.1). There was an inverse correlation between tubular diameter and tubular density ($r = -0.84$, $P < 0.001$).

3.4.2.3 Tubular morphology

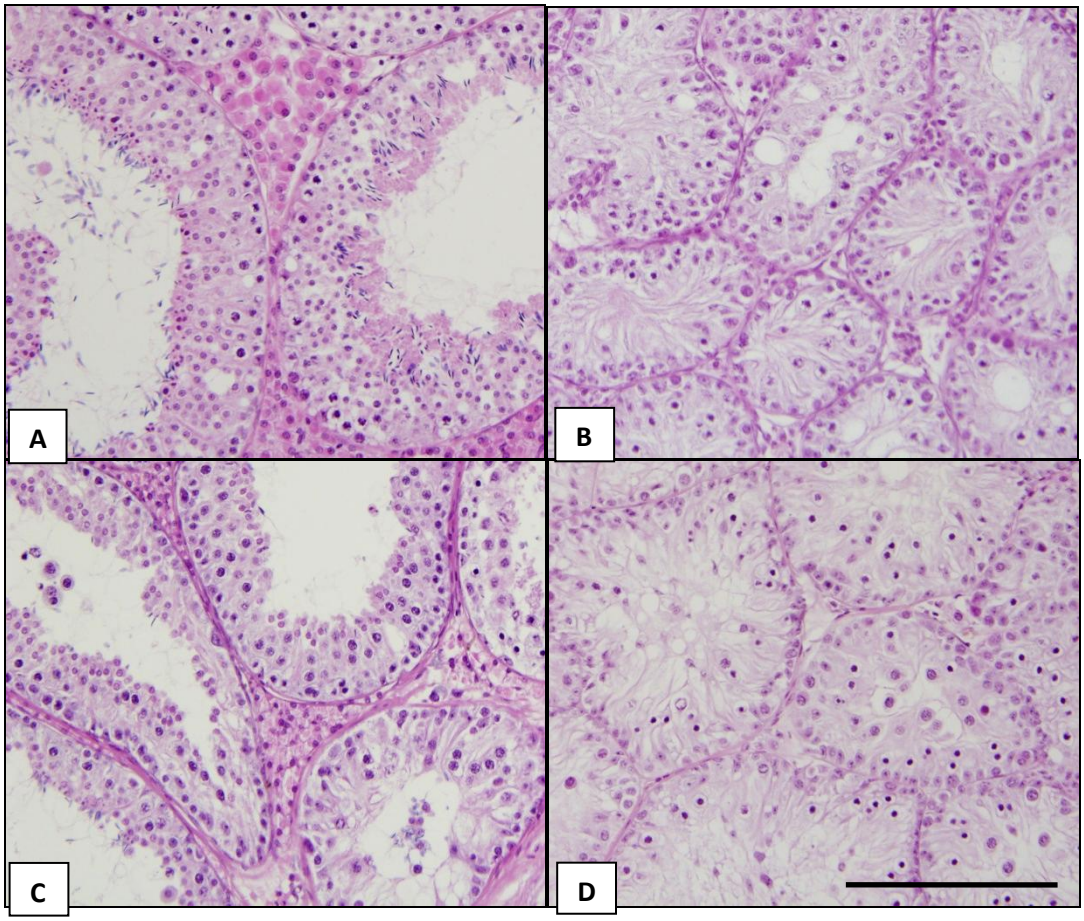
The donor testis tissue at the time of grafting contained seminiferous cords with Sertoli cells, gonocytes, and interstitial tissue with Leydig cells. The xenografts retrieved at 8

mo post-grafting displayed testicular development up to complete spermatogenesis. Representative histological appearance of testis tissue xenografts recovered from different groups of recipient mice are shown in Fig. 3.1.

Spermatozoa, as the most advanced germ cell type, were present among the seminiferous tubules of grafts from all groups. There was an effect of interaction between strain and gender ($P = 0.02$). The grafts from male SCID mice had the highest percentage of spermatozoa-containing tubules, followed by the group of male nude mice, while the groups of female SCID and nude recipients had the lowest percentages ($P < 0.05$, Table 3.1).

The percentage of seminiferous tubule cross-sections with elongated spermatids, as the most advanced germ cell type, was higher in grafts from the combined group of male than female mice ($13 \pm 1.4\%$ *vs.* $4 \pm 0.8\%$, $P < 0.001$, respectively). Among the four groups, grafts from the male SCID and nude mice had higher percentages of tubules with elongated spermatids than those of female mice ($P < 0.003$, Table 3.1).

Fig. 3.1 Histological appearance of testis tissue xenografts recovered from recipient mice at 8 mo post-grafting.



Xenografts from male SCID (A), female SCID (B), male nude (C), and female nude mice (D). The grafts from male SCID mice were larger and more developed than those of other groups. Scale bar = 200 µm.

The percentage of tubule cross-sections containing round spermatids was affected by gender only (10 ± 1.2% *vs.* 6 ± 1.1%, male *vs.* female, P = 0.006). The groups of male SCID and nude mice had grafts containing higher percentages of tubule cross-sections with round spermatids compared with those of the group of female nude mice (P = 0.01 , Table 3.1).

The percentage of tubule cross-sections containing spermatocytes, as the most advanced germ cell type present in the seminiferous tubules of xenografts differed depending on the mouse strain (27 ± 3.7% *vs.* 13 ± 2.3%, SCID *vs.* nude, P = 0.003), gender (10 ± 1.1% *vs.* 30 ± 3.5%, male *vs.* female, P = 0.001), as well as the interaction of strain and gender (P = 0.01). The highest percentage of tubules with spermatocytes was observed in grafts recovered from the group of female SCID mice, followed by those of female nude mice, while the groups of male SCID and nude mice had the least percentages (P < 0.05, Table 3.1).

The percentage of tubules with spermatogonia, as the only type of germ cell present, differed based on the mouse strain (8 ± 2.4% *vs.* 15 ± 3.6%, in SCID *vs.* nude groups, P = 0.02) as well as mouse gender (6 ± 2.4% *vs.* 17 ± 3.4%, male *vs.* female, P = 0.003). Among the four groups, the percentage of tubules containing spermatogonia was the highest in grafts from the group of female nude mice (P < 0.05, Table 3.1).

The percentage of tubule cross-sections showing Sertoli-cell-only in grafts was affected only by gender and was lower in the combined groups of male than female recipient

mice (12 ± 2.2% *vs.* 26 ± 3.8%, P = 0.002, respectively). The group of female SCID mice had higher percentage of tubules in this category than those of male SCID mice (P = 0.007, Table 3.1).

The percentage of degenerated seminiferous tubules in the recovered xenografts differed as a result of mouse strain, with the combined groups of SCID mice showing lower values than those of nude mice (11 ± 1.9% *vs.* 19 ± 2.5%, P = 0.04, respectively). However, among the four groups, no group could be singled out as being different (P > 0.05, Table 3.1).

Correlations between each of the above-mentioned outcomes of TTX and the average graft weight, total graft weight or the mouse body weight are shown in Table 3.2. There was a positive and significant correlation between the mouse body weight and the average graft weight. Testis weight was positively correlated with gross and histological development of the recovered grafts and negatively correlated with the percentage of tubules with spermatocytes, spermatogonia, and Sertoli-cell-only. Mouse body weight was positively correlated with tubular diameter and the percentage of tubules with spermatocytes, and negatively correlated with the percentage of tubules containing only Sertoli cells (Table 3.2).

Table 3.2 Correlations between mouse body weight or mean graft weight with characteristics of testis tissue xenografts recovered from recipient mice at 8 mo post-grafting.

	Mouse body weight (g)		Average graft weight (mg)	
	r	P	*r*	P
Mouse body weight (g)	-	-	0.4	0.01
Graft recovery rate (%)	0.2	0.2	0.4	0.01
Average graft weight (mg)	0.4	0.01	-	-
Tubular diameter (µm)	0.4	0.02	0.5	0.0003
Tubular density (/mm^2)	-0.2	0.2	-0.3	0.1
Spermatozoa (% of tubules)	0.3	0.03	0.8	0.0001
Elongated spermatids (% of tubules)	0.2	0.1	0.7	0.0001
Round spermatids (% of tubules)	0.3	0.1	0.6	0.0001
Spermatocytes (% of tubules)	-0.03	0.8	-0.3	0.04
Spermatogonia (% of tubules)	-0.2	0.2	-0.6	0.0001
Sertoli-cell-only (% of tubules)	-0.4	0.02	-0.5	0.0002
Degenerated (% of tubules)	0.005	0.97	-0.005	0.97

Pearson's correlation coefficients (*r*) were calculated for the combined data from different groups of recipient mice grafted with fragments of porcine testis tissue, and evaluated at 8 mo post-grafting for spermatogenic development. Correlation coefficients were considered significant when $P < 0.05$.

3.5 Discussion

Xenografting of testis tissue has provided an experimental model to study testicular development and spermatogenesis in testis tissues from diverse donor species after grafting into recipient mice (Paris and Schlatt 2007; Rodriguez-Sosa and Dobrinski 2009). Following the original description of procedures for successful TTX (Honaramooz *et al.* 2002), castrated male nude mice were used as recipients in the majority of studies; however, no study has systematically investigated the virtue of recipient mouse type for xenografting.

Overall, results suggest that SCID mice provide a more suitable recipient model than nude mice for TTX in terms of both gross and histological development of grafts. Although the overall graft recovery rate was high (94%), the combined group of SCID mice had a higher graft recovery rate than that of nude mice. SCID mice had allowed an overall greater physical growth of the xenografts, with an average graft being ~1.8-fold as large as that of nude mice. In terms of spermatogenic development, grafts from SCID mice tended to display higher levels of development than those of the nude mice. Overall, the grafts from the group of male SCID mice had consistently higher values (significantly/numerically) in indicators of advanced spermatogenic development among the four groups. Conversely, the indicators of limited spermatogenic development or tubular degeneration were higher in the grafts from the combined group of nude mice than in those of SCID mice.

The observed differences in the development of grafts between SCID and nude recipient mice may be due to their differing level of immunodeficiency, as also suggested for

engraftment of human cells (Ito *et al.* 2002), or due to other strain-related differences. Nude mice lack T lymphocytes and have no hair, whereas SCID mice lack both T and B lymphocytes but have a complete fur (Mueller and Reisfeld 1991; Kubota *et al.* 1993). SCID mice were originally used for TTX to avoid potential antibody immune responses when exogenous glycoprotein hormones were to be administered repeatedly to the recipient mice for extended periods of time (Honaramooz *et al.* 2004). To this date, a number of indirect comparisons of the outcome of TTX have been made between nude and SCID recipient mice, but no conclusive differences were found (Rathi *et al.* 2005; 2006; Geens *et al.* 2006). Therefore, our results provide the first definitive evidence for the superior potential of SCID mice as recipients for TTX.

Throughout the study, we observed indications that recipient mouse gender affects the outcome of TTX (significant effect for all examined criteria, except for body weight and the percentage of degenerated seminiferous tubules). Overall, ~10% more grafts were recovered from male than female recipient mice, and the grafts recovered from male recipients were ~3.7-fold heavier those from female recipients. When spermatogenic development of the grafts was compared between all male and female recipient mice, the grafts from male mice had a higher level of development than that of female mice. This included higher percentages of tubule cross-sections with indicators of advanced spermatogenesis in grafts from each combined group of male recipient mice than from their female counterparts (>5-fold, >3-fold and >1.5-fold, for tubules with spermatozoa, elongated spermatids and round spermatids, respectively, as the most advanced germ cells types). For the aforementioned categories, the highest percentages were also consistently found among the grafts from the groups of male mice. On the other hand,

the grafts recovered from the combined groups of female recipients had higher percentages (mostly by 1.5- to 3-fold) of tubule cross-sections with limited spermatogenic development or tubular degeneration than male recipients. The grafts from male recipient mice also had larger seminiferous tubular diameter and lower tubular density than those of female recipients. These results show that for gross and histological development of testis tissue xenografts, male recipient mice are more suitable recipients.

The recipient mice are usually gonadectomised at the time of grafting. Gonadectomy, by removing androgens/estrogens as the main source of negative feedback on pituitary gonadotropins allows the serum levels of gonadotropins to increase. This rise is perceived to be necessary or at least useful in providing a potential gonadotropic stimulation for development of the newly grafted testis tissue (Honaramooz *et al.* 2002; Schlatt *et al.* 2003; Dobrinski 2005; Paris and Schlatt 2007; Rodriguez-Sosa and Dobrinski 2009). However, no effect of gonadectomy was evident on any of the measured outcomes of TTX throughout the present study. Interestingly, female recipient mice, even those with intact ovaries, were fully capable of providing a supportive milieu for the developing testis tissue xenografts. The lack of a difference between intact and gonadectomised recipient mice for support of testis tissue xenografts may imply that the observed differences between male and female recipients could have been due to non-hormonal differences. This could be in part due differences in body weight between the genders since male mice tended to be on average heavier than female mice and there were positive significant correlations between the body weight and average graft weight and several of other measures of graft development. Nevertheless, the absence of a need

to perform gonadectomy on recipient mice should make the procedure for TTX easier and reduce potential surgical complications due to gonadectomy.

We also observed significant correlations between the average graft weight and most TTX endpoints in the present study. Generally, larger grafts tended to be more developed than smaller grafts.

Overall, male SCID mice are the most suitable recipient for TTX, and they do not need to be castrated for optimal results.

CHAPTER 4: THE NUMBER OF GRAFTED FRAGMENTS AFFECTS THE OUTCOME OF TESTIS TISSUE XENOGRAFTING IN RECIPIENT MICE[2]

4.1 Abstract

Testis tissue xenografting (TTX) into immunodeficient mice has provided a novel *in vivo* model for the study of testis function from diverse species in a laboratory model. The objective of this study was to evaluate the effect of the number donor testis tissue fragments on the outcome of TTX. Small fragments of testis tissue from piglet donors were grafted subcutaneously under the back skin of recipient mice, each mouse received 2, 4, 8 or 16 fragments (n = 10 mice/group). At 8 mo post-grafting, the recipient mice were sacrificed and the recovered grafts were evaluated for growth and development. The mean graft recovery rate was ~94% (range 86-98%) which did not differ among the groups ($P > 0.05$). Both the total and mean weights of recovered grafts from recipient mice that received 16 testis tissue fragments were higher than those in mice that received 2 fragments ($P < 0.05$). The mice with 16 grafts had the highest relative weight of the vesicular gland (index) at the time of sacrifice; the lowest vesicular gland weight was seen in the group of mice that received 2 fragments ($P = 0.007$). The grafts in the mice with 16 testis tissue fragments had a higher percentage of tubules with round spermatids than the group of mice that received 2 fragments ($P < 0.05$). The percentage of degenerated seminiferous tubules in the xenografts was higher in the groups of mice grafted with 2 fragments ($P = 0.02$). We also observed significant correlations between the average graft weight and most endpoints examined in the present study. Generally,

[2] Manuscript has been accepted for publication in *Veterinary Medicine International.*

heavier grafts were more developed than smaller grafts. These results suggest that as many as 16 tissue fragments can be grafted into immunodeficient mice for optimal results.

4.2 Introduction

The neonatal testis contains immature interstitial Leydig cells and seminiferous cords enclosing gonocytes, the only germ cell type present and precursors of SSCs, as well as immature somatic Sertoli cells. During prepubertal development, the testis tissue undergoes a number of developmental changes to transform into a mature tissue capable of androgen release and spermatogenesis, a organised cyclic process that continuously produces haploid spermatozoa from diploid male germline cells (Russell *et al.* 1990). The underlying mechanisms controlling this process, especially the maturational changes in the testis of humans and large animals, are difficult to study *in situ* and it has been suggested that these events can be more appropriately studied in an experimental model (Gassei and Schlatt 2007; Huleihel *et al.* 2007).

In the absence of an *in vitro* model that can repeatedly replicate testicular maturation and spermatogenesis (Parks *et al.* 2003), testis tissue xenografting (TTX) has emerged as a means to overcome these limitations by providing an *in vivo* culture system. Using TTX, small fragments of testis parenchyma from an immature donor of any mammalian species are grafted under the back skin of immunodeficient mice (Honaramooz *et al.* 2002; Schlatt *et al.* 2002). This strategy maintains the structural integrity and cell associations needed for the subsequent cellular development of spermatogenesis. Xenografting of testis tissue has been successful in inducing maturation in the tissue and

development leading up to complete spermatogenesis using a wide range of immature donor species including laboratory, farm or companion animals and primates in a recipient mouse model (Honaramooz *et al.* 2002; 2004; Schlatt *et al.* 2002; 2006; Snedaker *et al.* 2004; Oatley *et al.* 2004; Rathi *et al.* 2005; 2006; Abrishami *et al.* 2010a). Xenogeneic spermatozoa have been retrieved from testis tissue xenografts by mechanical dispersion and used for intracytoplasmic spermatozoa injection (ICSI) to confirm fertilization competence or to produce offspring (Honaramooz *et al.* 2002; 2008; Shinohara *et al.* 2002; Schlatt *et al.* 2003; Ohta *et al.* 2005; Jahnukainen *et al.* 2006; Nakai *et al.* 2010). TTX has been shown to have several important applications such as allowing the study and manipulation of spermatogenesis, providing insights into testis function, and offering a new option for male germline preservation especially from neonatally lethal phenotypes and rare or endangered animals that die before puberty (Ohta *et al.* 2005; Jahnukainen *et al.* 2006; Paris and Schlatt 2007; Arregui *et al.* 2008a; Rodriguez-Sosa and Dobrinski 2009; Abrishami *et al.* 2010a).

Following the methodology described in the first reports of successful TTX (Honaramooz *et al.* 2002; Schlatt *et al.* 2002), most researchers have used eight testis tissue fragments, of ~1 mm^3 (or ~5 mg) each, to graft under the back skin of recipient mice. This number of grafts per mouse was chosen for practical reasons as four grafts could be easily grafted on either side of the midline and spaced out between the neck and sacral region. It is desirable to maximise the mass of harvested grafts; however, no study has systematically evaluated the optimum number of tissue fragments that can result in the highest growth and development of the grafted testis tissue. Therefore, the

objective of this study was to determine the effect of the initial number of testis tissue fragments grafted per recipient mouse on measured outcomes of TTX.

4.3 Materials and Methods

4.3.1 Study design

We examined the effect of using different numbers of testis tissue fragments per recipient mouse on the outcome of TTX. Groups of castrated immunodeficient nude mice (n = 10 mice/group) received 2, 4, 8 or 16 fragments of donor testis tissue under the back skin. At 8 mo post-grafting, the recipient mice were sacrificed and the visible xenografts were recovered. The retrieved xenografts were then evaluated for graft weight, recovery rate and histological development. The weight of the vesicular glands in the recipient mice was also measured as an indication of androgen production by the testis grafts. Experimental procedures involving animals were approved by the University of Saskatchewan's Institutional Animal Care and Use Committee.

4.3.2 Donor testis tissue

Donor testis tissue was obtained through aseptic castration of 1-wk-old Yorkshire-cross piglets (Camborough-22 × Line 65, PIC Canada Ltd., Winnipeg, MB, Canada) at a swine facility. Immediately after castration, the testes were immersed in ice-cold Dulbecco's phosphate buffered saline (DPBS, Cat. No. 20-031-CV, Mediatech, Manassas, VA, USA) containing 2% w/v antibiotic/antimycotic solution (Cat. No. 30-004-CI, Mediatech). The testes were transferred to the laboratory within 1 h, where they

were rinsed three times with DPBS and the tunica albuginea, rete testis and overt connective tissues were removed. Small fragments of testis, approximately 5 mg in weight or 1 mm^3 in volume, were cut from the testis parenchyma using a scalpel and maintained in Dulbecco's modified Eagle's medium (DMEM, Cat. No. 10-013-CM, Mediatech) on ice until grafting within 2 h. As a reference for graft development, samples of the fragments were fixed in Bouin's solution overnight, washed with and kept in 70% v/v ethanol and processed for histology.

4.3.3 Recipient mice and procedures for xenografting of testis tissue

Male immunodeficient nude mice (NCr, nu/nu, Taconic, Germantown, NY, USA) were randomly assigned to one of four groups (n = 10 mice/group) to receive 2, 4, 8 or 16 testis tissue grafts. At the time of grafting, mice were ~10 wk old and maintained aseptically in groups of 10 in perplexiglass microinsulators in a room with controlled photoperiod (lights on from 0600 through 1800). The mice were provided with sterile water and mouse chow *ad libitum*. At the time of surgery, the mice were anesthetised with intra-peritoneal injection of ketamine hydrochloride (100 mg/kg; Ketalene, Bimeda-MTC, Cambridge, ON, Canada) and xylazine hydrochloride (10 mg/kg; Vet-A-Mix, Shenandoah, IA, USA). All recipient mice underwent castration using an abdominal midline approach. A transverse linear incision (~5 mm in length) per planned graft was made into the back skin of each mouse, a small subcutaneous pouch was created using blunt dissection and a testis tissue fragment was placed in the subcutaneous pouch. Wound clips were used to close the incisions (Michel Clips 7.5 mm, Miltex, York, PA, USA).

4.3.4 Gross and histological analysis

At 8 mo post-grafting, the mice were anesthetised, sacrificed and weighted. Visible xenografts were retrieved, weighted and fixed in Bouin's solution overnight. The testis tissue xenografts were washed with and kept in 70% ethanol solution prior to histological processing. After processing, the grafts were paraffin blocked and sectioned (at 6 µm thickness) at the largest diameter. The tissue sections were stained with haematoxylin and eosin evaluated under light microscopy equipped with a digital camera.

From each mouse as the experimental unit, 16 digital micrographs were captured from randomly selected areas of the sectioned xenografts so that each graft was represented in at least one micrograph. A calibrated microscope was used to capture the micrographs at 200× magnification which were then given codes that were unknown to the operator analysing them. All seminiferous tubules (or up to 200, if there were more) were analysed in each micrograph (Image pro Express, version 6.0.0.319 for Windows XP/Professional, Media Cybernetics, Inc, MD, USA). The endpoints for evaluation included the number of tubule cross-sections to calculate the tubular density (per mm^2), tubular diameter in the widest cross-section, the morphology of seminiferous tubules and the most advanced germ cell type in the tubules.

To evaluate the morphology of the seminiferous tubules and the most advanced germ cell type, the presence of: 1) mature spermatozoa in the lumen of the seminiferous tubule cross-section; 2) elongated spermatids as the most advanced germ cells; 3) round spermatids as the most advanced germ cells; 4) spermatocytes as the most advanced

germ cells; 5) spermatogonia as the only type of germ cells; 6) Sertoli-cell-only tubule; and 7) complete tubular degeneration or fibrosis we recorded.

The vesicular glands were also dissected out and weighed to calculate the vesicular gland index. The index (% of the vesicular gland weight/body weight) has been used as an indicator of the levels of bioactive androgens released by the xenografts (Honaramooz *et al.* 2002; Schlatt *et al.* 2003).

4.3.5 Statistical analysis

Since the recipient mouse was considered an experimental unit, the data obtained from all testis tissue xenografts within a mouse were pooled. The evaluated endpoints were: the mouse body weight (g), graft recovery rate (% of visible grafts retrieved compared with the original number of fragments grafted), total and average graft weight (mg), vesicular gland index (%), seminiferous tubule diameter (μm), tubular density (/mm^2), the percentages of tubule cross-sections with spermatozoa, elongated spermatids, round spermatids, spermatocytes, or spermatogonia as the most advanced germ cell type, in addition to the percentages of seminiferous tubules containing Sertoli-cell-only or showing degeneration or fibrosis.

The data were analysed for the effect of the number of testis tissue fragments using ANOVA. The Pearson product-moment correlation coefficients were calculated to determine the relationship between mouse body weight and graft weight with the above-mentioned endpoints. Data are expressed as means ± SEM and $P < 0.05$ was considered significant.

4.4 Results

4.4.1 Mouse body weight, graft weight and graft recovery rate

Of 40 recipient mice, 3 died before the scheduled time of sacrifice and were excluded from data analysis. In the remaining 37 mice, body weight did not differ among groups ($P = 0.2$) and a total of 275 out of 294 grafts were recovered (overall, 94% graft recovery). The graft recovery rate, ranging from 86% to 98%, and did not differ among the four groups of recipient mice ($P = 0.7$, Table 4.1).

At 8 mo post-grafting, the average weight of recovered grafts in all groups had increased, compared with the original weight of 5 mg each ($P = 0.008$), with the group of mice receiving 16 testis tissue fragments showing the highest and the group of mice receiving two fragments showing the lowest average graft weight ($P = 0.02$, Table 4.1). The total weight of grafts per recipient mouse was higher in the groups of mice that received 16 or 8 fragments than the groups of mice that received 4 or 2 fragments ($P < 0.001$, Table 4.1).

The relative weight of the vesicular gland (index) at the time of sacrifice was highest in the group of mice receiving 16 testis tissue fragments and lowest in the group of mice receiving two fragments ($P = 0.007$, Table 4.1).

Table 4.1 Characterisation of testis tissue xenografts recovered from recipient mice at 8 mo post-grafting.

	Number of testis tissue fragments grafted (per mouse)			
	2	4	8	16
Number of mice analysed*	8	10	9	10
Mouse body weight (g)	29 ± 0.5	29 ± 1.5	30 ± 0.6	32 ± 0.9
Graft recovery rate (%)	86 ± 9.2	98 ± 2.5	90 ± 8.7	95 ± 3.8
Average graft weight (mg)	106 ± 38.0^{a}	196 ± 25.5^{ab}	208 ± 36.6^{ab}	278 ± 39.4^{b}
Total graft weight (mg)	192 ± 76.2^{a}	704 ± 82.2^{a}	1,619 ± 289.9^{b}	2,443 ± 338.8^{b}
Vesicular gland index (%)	0.1 ± 0.06^{a}	0.3 ± 0.06^{ab}	0.3 ± 0.05^{ab}	0.5 ± 0.06^{b}
Tubular diameter (µm)	126 ± 18.2	121 ± 14.7	141 ± 9.1	144 ± 2.6
Tubular density (/mm^2)	31 ± 6.4	39 ± 3.4	38 ± 3.1	32 ± 1.6
Spermatozoa (% of tubules)	17 ± 7.5	23 ± 6.3	26 ± 6.1	28 ± 3.2
Elongated spermatids (% of tubules)	4 ± 1.9	10 ± 2.0	8 ± 2.1	11 ± 1.6
Round spermatids (% of tubules)	3 ± 1.3^{b}	8 ± 2.2^{ab}	8 ± 1.9^{ab}	11 ± 1.5^{a}
Spermatocytes (% of tubules)	4 ± 2.4	16 ± 3.4	13 ± 2.3	12 ± 2.7
Spermatogonia (% of tubules)	4 ± 1.9	5 ± 1.4	4 ± 1.6	3 ± 2.4
Sertoli-cell only (% of tubules)	42 ± 8.8	28 ± 6.4	29 ± 6.9	21 ± 3.0
Degenerated (% of tubules)	27 ± 5.7^{a}	11 ± 3.3^{b}	12 ± 1.6^{b}	14 ± 2.6^{ab}

Four groups of recipient mice were grafted with different numbers of porcine testis tissue fragments, and the xenografts were evaluated at 8 mo post-grafting for spermatogenic development. Recipient mice were gonadectomised male nude mice and randomly assigned into groups that received 2, 4, 8 or 16 testis tissue fragments per mouse (n = 10 mice/group). * The number of recipient mice per group that survived to the time of analysis at 8 mo post-grafting (the original number was 10 mice per group). Graft recovery rate was defined as the relative number of visible xenografts retrieved compared with the number of fragments grafted. The average graft weight and histological evaluations were based on the recovered grafts. The average seminiferous tubule diameter and tubular density (the number of the seminiferous tubules/mm^2) were calculated in the largest cross-section of the recovered grafts. For comparison, at the time of grafting, the donor tissue had seminiferous cords that were 47 ± 1.1 µm in diameter and at a density of 95 ± 6.1 tubules/mm^2. Data are presented as mean ± SEM. ^{ab} Values with different superscript letters within each row are significantly different ($P < 0.05$).

4.4.2 Histological evaluation of the recovered testis grafts

4.4.2.1 Tubular diameter

The seminiferous cords diameters in newborn donor testis tissue was 47 ± 1.1 µm at the time of grafting. The tubular diameter in the grafts examined at 8 mo post-grafting did not differ among the groups of recipient mice that received different numbers of testis tissue fragments (P = 0.4, Table 4.1).

4.4.2.2 Tubular density

Neonatal donor testis tissue had a seminiferous tubular density of 95 ± 6.1 tubule cross-sections/mm^2 at the time of grafting. Tubular density of the grafts recovered from recipient mice after 8 mo did not differ among the groups (P = 0.3, Table 4.1).

4.4.2.3 Tubular morphology

At the time of grafting, the histology of neonatal donor testes showed interstitial tissue containing Leydig cells and seminiferous cords containing somatic Sertoli cells and gonocytes. At 8 mo post-grafting, the retrieved xenografts had complete spermatogenesis, comparable to the expected status in age-matched pig testes. Representative histological photomicrographs of the recovered testis tissue xenografts

from different groups of recipient mice are shown in Fig. 4.1. The most advanced germ cell type and the morphology of the seminiferous tubule cross-sections were evaluated in the retrieved xenografts and the results were compared among groups as follows:

Spermatozoa were observed within the lumen of seminiferous tubule cross-sections in the testis tissue xenografts from the majority of the recipient mice in all four groups (the exceptions were grafts from one or two recipient mice in groups of mice that received 2, 4 or 8 fragments at the time of grafting). The percentage of graft tubule cross-sections containing spermatozoa, as the most advanced germ cell types present, did not differ among the four groups ($P = 0.6$), and that of elongated spermatids only tended to differ among the groups ($P = 0.06$, Table 4.1).

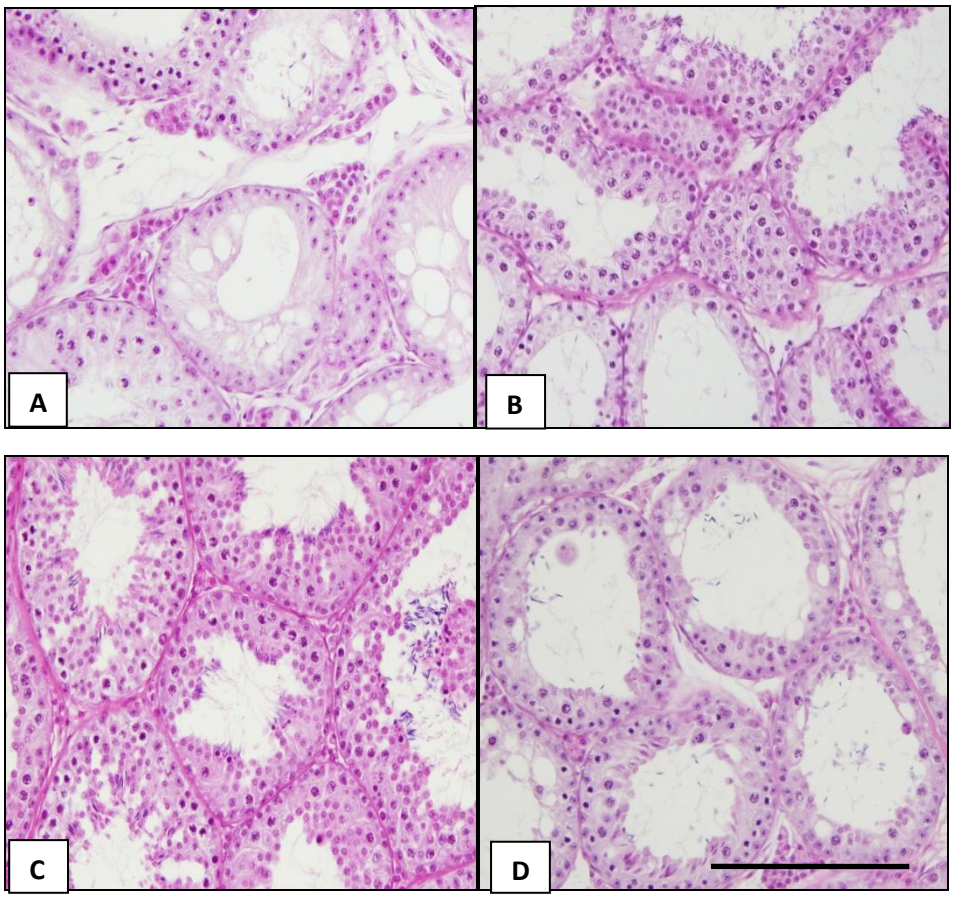
The percentage of seminiferous tubules with round spermatids, as the most advanced germ cell type, differed among groups ($P = 0.03$), with higher values observed in grafts from the group of mice that received 16 testis tissue fragments, compared with those that received two fragments ($P < 0.02$, Table 4.1).

The difference in the percentage of graft tubular cross-sections with spermatocytes, as the most advanced types of germ cell present, only approached significance ($P = 0.06$), and those of the tubules with spermatogonia or only Sertoli cells did not differ among the four groups ($P = 0.97$, 0.2, respectively, Table 4.1).

The percentage of degenerated seminiferous tubules in the xenografts varied among the groups ($P = 0.01$), with those in the group of mice receiving two testis tissue fragments

showing higher percentage of degenerated tubules than the groups of mice grafted with four or eight fragments (P = 0.02, Table 4.1).

Fig. 4.1 Histological photomicrographs of testis tissue xenografts recovered from recipient mice at 8 mo post-grafting.



Xenografts from recipient mice that received 2 (A), 4 (B), 8 (C) or 16 (D) testis tissue fragments. The grafts from mice receiving 16 fragments were larger and more developed than those from mice receiving 2 fragments at the time of grafting. Scale bar = 200 µm.

Table 4.2 summarizes the results of correlation analysis between each of the measured outcomes of TTX and the average graft weight, total graft weight or the mouse body weight. No significant correlations were found between the mouse body weight and the average or total graft weight. When correlations were present for the average graft weight and the different parameters of grafting outcome, they were positive and only negative for the percentages of tubules with spermatogonia or Sertoli-cell-only. A similar pattern of correlations was observed for those of the total graft weight. The mouse body weight was positively correlated with tubular diameter and negatively correlated with the percentage of tubules containing only Sertoli cells.

Table 4.2 Correlations between mouse body weight, average graft weight or total graft weight and characteristics of testis tissue xenografts at the time of sacrifice at 8 mo post-grafting.

	Mouse body weight (g)		Average graft weight (mg)		Total graft weight (mg)	
	r	P	*r*	P	*r*	P
Mouse body weight (g)	-	-	0.2	0.3	0.3	0.1
Graft recovery rate (%)	-0.02	0.9	0.3	0.1	0.3	0.1
Average graft weight (mg)	0.2	0.3	-	-	0.8	0.0001
Total graft weight (mg)	0.3	0.1	0.8	0.0001	-	-
Tubular diameter (µm)	0.5	0.002	0.3	0.1	0.3	0.1
Tubular density (mm^2)	-0.1	0.4	-0.01	0.9	-0.04	0.8
Vesicular gland index (%)	0.1	0.4	0.4	0.02	0.4	0.02
Spermatozoa (% tubules)	0.07	0.1	0.5	0.01	0.5	0.001
Elongated spermatids (% tubules)	0.3	0.1	0.5	0.002	0.7	0.0001
Round Spermatids (% tubules)	0.3	0.07	0.5	0.001	0.6	0.0004
Spermatocytes (% tubules)	0.2	0.3	-0.1	0.6	0.01	0.96
Spermatogonia (% tubules)	-0.01	0.97	-0.5	0.01	-0.4	0.01
Sertoli-cell-only (% tubules)	-0.4	0.02	-0.5	0.01	-0.6	0.0002
Degenerated (% tubules)	0.1	0.6	-0.2	0.3	-0.3	0.1

Pearson's correlation coefficients (*r*) were calculated for the combined data from four different groups of recipient mice grafted with different numbers of porcine testis tissue fragments, and the grafts evaluated at 8 mo post-grafting for spermatogenic development. Correlation coefficients were considered significant when $P < 0.05$.

4.5 Discussion

Xenografting of testis tissue has provided a novel *in vivo* culture system to study testis function and to preserve spermatogenic potential of immature donors from diverse mammalian species in a laboratory mouse (reviewed by Paris and Schlatt 2007; Rodriguez-Sosa and Dobrinski 2009). However, little research has been dedicated to re-examine the methodology used to ensure optimal efficiency of the system in expanding the donor tissue. This study represents the first systematic examination of the optimal number of testis tissue fragments for xenografting into recipient mice.

Piglet testes were used as a source of donor tissue since the outcome of porcine TTX is well-established (Honaramooz *et al.* 2002; 2008, Abrishami *et al.* 2010b). We showed previously that piglet testes from our source (a university-affiliated swine farm) are a homogenous supply of testis tissue (Yang *et al.* 2010c). Castrated male nude mice were used as recipients for TTX based on previous studies (Honaramooz *et al.* 2002; Schlatt *et al.* 2002; Dobrinski and Rathi 2008). We also used testis tissue fragments that were of the same size (~1 mm^3 or ~5 mg) as those in the majority of previous reports, but used 2, 4 or 16 donor fragments as compared with the conventional number of 8 fragments.

In the present study, the rate of graft recovery did not differ among the four groups of recipient mice (range, 86-98%). However, the total weight of the recovered grafts was ~12-fold higher in the group of mice that received 16 fragments than the group that received two fragments, and interestingly, the average graft weight was also ~2.5-fold

higher in the 16-fragment group compared to the 2-fragment group of mice. This demonstrated that the recipient mouse is fully capable of supporting the development of a much larger mass of grafted donor testis tissue than previously used.

In a recent report using TTX with donor lamb testes tissue (Rodriguez-Sosa *et al.* 2010), two flat strips of testis tissue (~9×5×1 mm or ~45 mm^3 each) were grafted per recipient mouse and recovered after 4 mo resulting in a 2.4-fold growth in the size of grafts. Although a direct comparison with the conventional size of testis fragments was not made, it may be deduced that the relative physical growth of the flat stripes of testis tissue per mm^3 was much less than that of cube fragments used in our study. In other words, a 45 mm^3-flat stripe grew by ~2-fold on average in that study, while the 1 mm^3-cube fragment in our study grew on average by ~21, 39, 41, or 55-fold (in 2, 4, 8 or 16-fragement groups, respectively). Similarly, the expansion of the total mass of grafted tissue was also much higher using our small fragments than using flat stripes in the above study. For instance, given the available data from the two studies, even 8 mm^3 (8 × 1 mm^3) total mass of small cube fragments grafted per mouse in our study resulted in a greater expansion of the tissue (to ~323 mm^3 or ~1,619 mg) than the 90 mm^3 (2 × 45 mm^3) flat stripes (to ~216 mm^3 or ~1,080 mg). In the above study (Rodriguez-Sosa *et al.* 2010), it was also concluded that the percentage of grafts that survived and percentage of seminiferous tubules that underwent spermatogenesis were the same as those reported after xenografting small fragments of ovine testis tissue (Arregui et al 2008a). The differential rate of growth between our observations and those of the above study may be more due to the differences in shape of the grafted tissue, where small fragments may allow a greater degree of vascularisation (Schmidt *et al.* 2006b; Schlatt *et al.* 2010).

Differences in the species- or sampling times cannot be ruled out in explaining these observations; however, in our experience with ovine testis tissue (Arregui et al 2008a), these grafts reach physical growth and spermatogenic development earlier than those of the porcine tissue grafts and by 4 mo post-grafting. Therefore, the use of small fragments of testis tissue (of ~1 mm^3) is recommended, especially if the goal of TTX is to expand a limited amount of testicular tissue, such as when the donor is a rare or endangered immature individual that has died unexpectedly.

The accessory sex glands, particularly the vesicular glands in mice, are highly androgen-dependent and will regress to less than 10% of their normal weight (130-340 mg) within a few wk after castration (Schlatt *et al.* 2003). This process is reversible and therefore, measuring the weight of the vesicular glands in recipient mice castrated prior to TTX can be used as an assay to evaluate the production of bioactive androgens by the grafted testis tissue (Honaramooz *et al.* 2002; Schlatt *et al.* 2003). In the current study, the vesicular gland indices in the group of mice that received 16 fragments were ~5-fold higher than mice that received two fragments (0.5% *vs.* 0.1%, respectively), likely as a consequence of higher mass of the testis tissue grafts capable of producing androgens. For comparison, the vesicular gland index in an intact 30-gram nude mouse is 0.4% to 1.1% (Schlatt *et al.* 2003).

In the present study, spermatogenic development also differed among the groups since grafts from the group of mice receiving 16 fragments had ~3-fold more tubules with round spermatids (as the most advanced germ cell type) than the group of mice receiving two fragments. The grafts from the group of mice that received two fragments also had

about one-third the percentages of tubule cross-sections with elongated spermatids or spermatocytes, but ~2-fold higher percentage of degenerated tubules than the other groups. These results indicate that the grafts from the group of mice that received 16 testis tissue fragments not only yielded more graft tissue, collectively and individually, but grafts were also more developed. This may be due to the higher levels of androgen produced from grafts in this group of recipient mice which might have had a positive effect on the gross and histological development of single grafts. This conclusion is also consistent with the results of the correlation analysis in which higher average or total graft weights were positive indicators of graft development.

We also observed significant correlations between the average graft weight and most TTX endpoints examined in the present study. Generally, larger grafts were more developed than smaller grafts.

The current study provided the first systematic evaluation of the effect of changing the number of tissue fragments on the outcome of TTX using a donor porcine model. This study demonstrated that total number of donor testis tissue fragments can be as many as 16 fragments for optimal results.

CHAPTER 5: TESTIS TISSUE XENOGRAFTING FROM IMMATURE BISON AND DEER DONORS INTO RECIPIENT MICE TO DETERMINE THE FEASIBILITY OF THIS STRATEGY FOR SALVAGING GENETICS FROM ENDANGERED UNGULATES[3]

5.1 Abstract

Testis tissue collected after the unexpected deaths of two newborn bison calves (*Bison bison bison*) and a 2-mo-old white-tailed deer fawn (*Odocoileus virginianus*) were grafted into recipient mice. The objective was to evaluate the long-term outcome of testis tissue xenografting (TTX) from bison and deer as models for closely-related rare or endangered ungulates. Small fragments of donor testis tissue (~5 mg) were grafted under the back skin of immunodeficient recipient mice (n = 15 mice for bison xenografts and n = 7 mice for deer xenografts; 8 fragments/mouse). Single xenograft samples were removed from representative recipient mice every 2 mo from grafting for up to 16 mo for bison xenografts, and 14 mo post-grafting for deer xenografts. The retrieved xenografts were evaluated for seminiferous tubular density (per mm^2), tubular diameter and morphology and identification of the most advanced germ cell type present in each tubule cross-section. Overall, 69% of the grafted testis fragments from donor bison and 63% of those from donor deer were recovered as xenografts. The weight of bison testis tissue xenografts increased ($P < 0.02$) ~4-fold by 2 mo and ~10-fold by 16 mo post-

[3] Manuscript has been submitted for publication in *Theriogenology.*

grafting. For bison grafts, a donor effect on efficiency of spermatogenesis was observed. Gradual maturational changes were evident in the form of seminiferous tubule expansion starting at 2 mo, first appearance of spermatocytes at 6 mo, round spermatids at 12 mo, and elongated spermatids at 16 mo post-grafting. Testis tissue xenografts from donor white-tailed deer also showed a gradual testicular development starting with tubular expansion by 2 mo and presence of spermatocytes by 6 mo post-grafting, round and elongated spermatids by 8 mo, followed by fully-formed spermatozoa by 12 mo post-grafting. The timing of complete spermatogenesis roughly corresponded to the reported timing of sexual maturation in these species. This study demonstrated, for the first time, that TTX from neonatal/immature donor bison or deer into immunodeficient recipient mice can result in testicular tissue maturation and spermatogenesis in the grafts up to the stage of spermatozoa production. These results provide a model for salvaging genetic material from immature males of rare or endangered ungulates that die prematurely.

5.2 Introduction

Species extinction rates seem to have accelerated in recent decades (Lenzen *et al.* 2009). The proportion of threatened species is much higher in mammals than that in other groups such as birds (Mace *et al.* 2008) and currently an alarming 23% of all mammalian species are considered threatened or vulnerable. Among mammals, ungulates are one of the groups with high percentage of threatened species. It is believed that ungulates are the only mammalian group in which hunting is a more frequent cause of threat than loss of habitat (IUCN 2006, www.iucnredlist.org).

In valuable, rare or endangered species/breeds, to prevent the permanent loss of an adult male's potential contribution to the genetic variability, it is feasible to collect spermatozoa before or even shortly after death by retrieval from the ejaculate, epididymis or testes, and cryopreserve the spermatozoa for future use in assisted reproduction (Kishikawa *et al.* 1999; Gañán *et al.* 2009; Martínez *et al.* 2008). However, preservation of spermatozoa is not an option when young males die prior to reaching sexual maturity. Cloning has been used for a number of species and especially where the goal has been to produce a genetically exact replica of an individual. However, development of cloning for a new species is technically demanding and costly, but more importantly cloning does not immediately provide the genetic diversity that would otherwise be offered by gametes.

Grafting of testis tissue fragments from a donor male under the back skin of recipient mice provided a novel tool for the study, manipulation, and propagation of the male germline (Honaramooz *et al.* 2002). Successful development of functional spermatozoa in grafts of testis tissue from neonatal donors has been reported in a variety of species, including mice, hamsters, cats, dogs, pigs, goats, sheep, cattle, horses and primates, (Honaramooz *et al.* 2002; 2004; Oatley *et al.* 2004; Snedaker *et al.* 2004; Rathi *et al.* 2005; Rathi *et al.* 2006; Abrishami *et al.* 2010a). The spermatozoa recovered from such grafts have been shown to be fertilization competent after intracytoplasmic spermatozoa injection (ICSI) (Honaramooz *et al.* 2002; 2004; 2008), leading to the birth of healthy progeny (Schlatt *et al.* 2003; Nakai *et al.* 2010). Recently, we also developed methods for cool storage of immature testis tissue for up to 6 days and cryopreservation for long-term storage (Rathi *et al.* 2006; Honaramooz *et al.* 2008). TTX can provide a unique

solution for rescuing the genetic material of immature males that die prematurely, by producing spermatozoa in xenografts, followed by extraction and cryopreservation for future use in ICSI.

Wild ungulates have been a primary focus of research in wildlife management. The high incidence of carriers of serious zoonotic diseases (e.g., tuberculosis and brucellosis) among isolated groups of wild bison in western and northern Canada is a cause of great concern and a threat to the Canadian economy, health and wildlife conservation. Dealing with this situation has not been easy since drastic measures may end in loss of genetic diversity, and continuation of the status quo could have severe consequences (Kaneene and Pfeiffer 2006; Tessaro 1986; Wobeser 2009). Conventional reproductive technologies are being tested to ensure banking of safe sources of genetically-diverse gametes from the population of wild bison. However, development of novel strategies is required to conserve genetic diversity offered by bison calves that do not reach sexual maturity.

In North America, farming of bison and deer as specialised hoofstock is also expanding into a viable industry. Therefore, not only are bison and deer the subject of small but increasing number of investigations into the reproductive physiology and management, but also provide excellent models for research into reproductive strategies for conservation purposes aiming at endangered or threatened ungulates. Farming of bison, especially plains bison has seen considerable growth in recent years, owing to an increased international demand for bison meat (Firmage-O'Brien 2010). On the other

hand, farming of elk and deer is aimed at producing velvet antler for domestic or Asian market, or at selecting and propagating genetically superior bucks for sports outfitting.

Therefore, the objective of this study was to expand the application of TTX utilizing bison calves and deer fawns as models for preservation of the genetic diversity offered by individual rare or endangered ungulates that die prematurely.

5.3 Materials and Methods

5.3.1 Study design

We examined the long-term outcomes of TTX from immature bison and white-tailed deer donors into recipient mice. Small fragments of testis tissues from two newborn bison calves and a 2-mo-old white-tailed deer fawn were grafted under the back skin of immunodeficient recipient mice. Single grafts were then removed surgically from representative mice every 2 mo after grafting, to measure long-term viability while probing into the developmental pattern of the xenografts. For bison xenografts, signle samples were taken every 2 mo until 16 mo post-grafting and for the white-tailed deer xenografts, sampling continued until 14 mo post-grafting. The recovered xenografts were assessed for physical and histological development. Experimental procedures involving animals were approved by the University of Saskatchewan's Institutional Animal Care and Use Committee.

5.3.2 Donor testis tissue

Donor testes were collected post-mortem from two newborn plains bison calves (*Bison bison bison*) at a university-affiliated research center. Bison calf No. 1 had died shortly after birth and the testes were collected and maintained at 4°C overnight, whereas bison calf No. 2 was euthanised because of respiratory distress and the testes were collected fresh and sent to our laboratory along with the overnight-preserved testes. Testes from a 2-mo-old white-tailed deer (*Odocoileus virginianus*) were also collected immediately after euthanasia because of serious skin lesions at a commercial deer farm. In both cases, the testes were transferred to the laboratory in ice-cold Dulbecco's phosphate buffered saline (DPBS, Cat. No. 20-031-CV, Mediatech, Manassas, VA, USA). The testes were then washed 3 times with DPBS, containing 2% w/v antibiotic/antimycotic solution (Cat. No. 30-004-CI, Mediatech), and the tunica albuginea, rete testis and overt connective tissue were removed. The testis parenchyma was then divided into small fragments of ~5 mg (or ~1 mm^3 in volume), and maintained in Dulbecco's modified Eagle's medium (DMEM, Cat. No. 10-013-CM, Mediatech) on ice until grafting into recipient mice within 2 h. Prior to grafting, representative donor testis tissue fragments were fixed in Bouin's solution overnight, washed with and kept in 70% v/v ethanol and processed for histology as a reference for graft development.

5.3.3 Recipient mice and procedures for xenografting of testis tissue

Recipient mice were gonadectomised immunodeficient nude mice (NCr, nu/nu, Taconic, Germantown, NY, USA) that were ~10 wk old at the time of grafting and maintained aseptically in groups of 3 or 4 in perplexiglass micro-insulators under controlled

photoperiod environment (lights on from 0600 through 1800) with sterile water and mouse chow provided *ad libitum*.

In preparation for surgery, the mice were anesthetised with intra-peritoneal injection of ketamine hydrochloride (100 mg/kg; Ketalene, Bimeda-MTC, Cambridge, ON, Canada) and xylazine hydrochloride (10 mg/kg; Vet-A-Mix, Shenandoah, IA, USA). Each mouse received 8 transverse linear incisions (~5 mm in length) into the back skin, 4 on each side of the midline. A small subcutaneous pouch was made through each incision and a testis tissue fragment was inserted and the incision was closed using wound clips (Michel Clips 7.5 mm, Miltex, York, PA, USA). In order to test the potential effect of donor in the bison group, four testis tissue fragments from bison calf No. 1 were grafted on the left side of the midline and four testis tissue fragments from bison calf No. 2 were grafted on the right side.

5.3.4 Gross and histological analysis

Representative mice from the group of recipients with bison testis tissue xenografts were anesthetised and underwent single-graft removal or were sacrificed at 2, 4, 6, 8, 10, 12, 14 and/or 16 mo post-grafting, at which time the sampling period ended and the mice were maintained for an additional 4 mo to provide more time for further potential development of the grafts (see Table 5.1). All remaining mice were sacrificed at 20 mo post-grafting and xenografts recovered.

Representative mice carrying white-tailed deer testis tissue xenografts were also selected for graft removal at 2, 4, 6, 8, 10 and/or 12 mo after grafting, and the remaining mice were sacrificed at 14 mo after grafting (see Table 5.3).

For both bison and deer groups, visible testis tissue xenografts from each time-point (see Tables 5.1 and 5.3) were dissected out and individually weighed. The retrieved grafts were fixed in Bouin's solution overnight, washed with and kept in 70% ethanol solution until processing for histology. The fixed tissues were then processed, paraffin blocked and sectioned (at 6 µm thickness) at the largest diameter of the graft. The sectioned tissues were stained with haematoxylin and eosin and analysed using light microscopy equipped with a digital camera.

For histological analysis of the xenografts, images were captured from all cross-sections of each xenograft. The images were taken at 200× magnification using a calibrated microscope and labelled anonymously, and the operator blindly analysed each image. All seminiferous tubule cross-sections within the images underwent morphometric measurements. The evaluation included counting tubule cross-sections to calculate the tubular density (per mm^2), measuring the widest tubular diameter using a software (Image pro Express, Media Cybernetics, Inc, MD, USA), and examining tubular morphology and determining the most advanced germ cell type in each tubule cross-section. Tubular morphology and the most advanced germ cell type present were categorised into: 1) gonocytes/spermatogonia present as the only type of germ cells; 2) spermatocytes present as the most advanced germ cells; 3) round spermatids present as the most advanced germ cells; 4) elongated spermatids present as the most advanced

germ cells; 5) mature spermatozoa present in the lumen of the seminiferous tubule cross-section; 6) Sertoli-cell-only tubule; and 7) complete tubular degeneration or fibrosis.

5.3.5 Statistical analysis

The data obtained from testis tissue xenografts collected from each mouse per time-point were averaged (and from each donor bison sample within the mouse). The endpoints considered in this study included the mean graft weight (mg), seminiferous tubule diameter (μm), tubular density (/mm^2), percentages of tubule cross-sections with gonocytes/spermatogonia, spermatocytes, round spermatids, elongated spermatids, and spermatozoa as the most advanced germ cell type, and the percentages of graft cross-sections displaying Sertoli-cell-only or degeneration.

For bison donor testis tissue xenografts, the data were analysed for the main effects of donor (2 donors) and time (8 time-points) and their interaction using two-way ANOVA (Sigmastat for Windows Version 3.5, Systat Software, San Jose, CA, USA). For deer testis tissue xenografts, the data were analysed for the effect of time using ANOVA. Percentages were transformed (using Arcsin function) prior to analysis using ANOVA.

The Pearson product-moment correlation coefficients were calculated between time and each of above-listed parameters of testis tissue graft development. Data are expressed as means ± SEM and $P < 0.05$ was considered significant.

5.4 Results

5.4.1 Bison testis tissue xenografts

Out of 15 recipient mice grafted with bison testis tissue, one mouse died before the scheduled time of sampling and was thus excluded from data analysis. From the remaining 14 mice, a total of 72 visible grafts (≥5 mg) were collected by the end of the sampling period at 16 mo post-grafting. An additional 5 grafts were collected at the time of sacrificing the remaining mice at 20 mo post-grafting. Therefore, in terms of the overall graft recovery rate, a total of 77 of 112 grafts were retrieved (69%). However, because the grafts recovered at the time of sacrifice were not part of the 2-mo interval sampling and did not show development beyond previous samples, they were not included in data analysis.

The results of TTX from the two donor bison calves into the same mice differed in some of the analysed endpoints; therefore, the summary of results from the two donor calves is presented separately in Table 5.1.

The overall average graft weight increased after grafting ($P = 0.02$), compared with the initial weight of each testis tissue fragment at the time of grafting (~5 mg). For instance, graft weight went up from an average of 20 mg for both donors at 2-mo post-grafting to 48 mg at 16 mo post-grafting. Although, the average graft weight did not change based on the donor calf or different time-points ($P > 0.05$, Table 5.1), a positive correlation ($r = 0.4$) existed between time as an overall factor and average graft weight, only for that of donor calf No. 1 ($P < 0.03$, Table 5.2).

5.4.1.1 Histological evaluation of the recovered testis grafts

5.4.1.1.1 Tubular diameter

At the time of grafting, the neonatal testis tissue from both donors had seminiferous cords that were 48 ± 1.1 µm in diameter. The tubular diameter in the grafts collected at 2 mo intervals post-grafting differed based on the overall effects of donor calf and time ($P = 0.02$ and $P < 0.001$, for donor and time-points, respectively, Table 5.1). Testis tissue xenografts from donor bison calf No. 1 (whose testis tissue was stored overnight at 4°C before grafting) had tubules that on average were 15 µm larger than those of donor calf No. 2 (whose testis tissue was used fresh) ($P < 0.05$, Table 1). Tubular diameters in grafts from donor bison calf No. 1 was ~68 µm at 2 mo post-grafting and ~142 µm at 16 mo post-grafting ($P < 0.05$, Table 5.1). These diameters for both donor calves were positively correlated with time ($r = 0.6$, $P < 0.003$, Table 5.2).

Table 5.1 Characterisation of bison testis tissue xenografts at different time-points after grafting.

	Donor calf	Time post-grafting (mo)							
		2	4	6	8	10	12	14	16
No. grafts analysed (No. mice) [§]	1	7 (2)	3 (1)	2	5 (3)	8	7	1	1
	2	8 (2)	4 (1)	2	4 (3)	9 (7)	7	3	1
Average graft weight (mg)	1	21 ± 7.2	17 ± 0.0	15 ± 4.0	24 ± 10.1	26 ± 3.9	35 ± 9.2	57 ± 0.0	41 ± 0.0
	2	18 ± 2.6	7 ± 0.0	31 ± 2.5	17 ± 5.4	21 ± 4.8	25 ± 5.4	37 ± 16.9	55 ± 0.0
Tubular diameter (µm)	1*	68 ± 1.1[a]	85 ± 0.0	97 ± 4.0	113 ± 9.1	104 ± 5.3	101 ± 7.9	132 ± 0.0	142 ± 0.0[b]
	2	60 ± 8.0	57 ± 0.0	98 ± 1.1	91 ± 5.8	88 ± 3.6	102 ± 10.5	98 ± 6.2	128 ± 0.0
Tubular density (/mm^2)	1	39 ± 6.4	24 ± 0.0	29 ± 4.6	24 ± 2.8	23 ± 1.4	24 ± 1.4	23 ± 0.0	20 ± 0.0
	2	45 ± 7.9[a]	28 ± 0.0	25 ± 9.1	29 ± 5.3	28 ± 1.5	26 ± 2.5	22 ± 2.0[b]	24 ± 0.0
Gonocytes or Spermatogonia (% of tubules)	1	27 ± 5.6	35 ± 0.0	0	4 ± 1.9	16 ± 4.7	8 ± 3.6	0	3 ± 0.0
	2	29 ± 6.5	9 ± 0.0	7 ± 1.3	5 ± 4.5	11 ± 4.9	16 ± 4.1	0	25 ± 0.0
Spermatocytes (% of tubules)	1*	0	0	42 ± 41.7	30 ± 18.3	1 ± 8.0	31 ± 9.6	1 ± 0.0	80 ± 0.0[*]
	2	0	0	32 ± 18.9	2 ± 1.0	2 ± 1.5	20 ± 7.3	1 ± 0.3	20 ± 0.0
Round spermatids (% of tubules)	1	0	0	0	0	0	1 ± 1.2	0	3 ± 0.0
	2	0	0	0	0	0	0.2 ± 0.2	0	0
Elongated spermatids (% of tubules)	1*	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0.8 ± 0.0[b*]
	2	0	0	0	0	0	0	0	0
Sertoli-cell only (% of tubules)	1	72 ± 5.6	59 ± 0.0	43 ± 31.3	46 ± 15.7	53 ± 6.3	46 ± 9.3	97 ± 0.0	9 ± 0.0
	2	71 ± 6.1	71 ± 0.0	33 ± 2.4	72 ± 6.2	69 ± 5.1	52 ± 7.9	61 ± 8.4	52 ± 0.0
Degenerated (% of tubules)	1	1.4 ± 0.1	7 ± 0.0	16 ± 10.4	20 ± 9.1	13 ± 2.7	14 ± 5.0	2 ± 0.0	4 ± 0.0
	2*	0.3 ± 0.3[a]	20 ± 0.0	28 ± 20.0	22 ± 9.3	10 ± 1.7	12 ± 3.1	39 ± 8.1[b*]	3 ± 0.0

A group of immunodeficient recipient mice was grafted with testis tissue fragments from two neonatal bison calves, 4 fragments from each donor were subcutaneously grafted on either side of the back of each mouse. The xenografts were sampled and evaluated at different time-points after grafting. The average graft weight and histological evaluations were based on the recovered grafts. The average seminiferous tubule diameter and tubular density (the number of the seminiferous tubules/mm^2) were calculated in the largest cross-section of the recovered grafts. For comparison, at the time of grafting, the donor tissue had seminiferous cords that were 48 ±

1.1 µm in diameter and at a density of 112 ± 6.1 tubules/mm^2. Data are presented as mean ± SEM. [ab] Values with different superscript letters within each row are significantly different ($P < 0.05$). * An asterisk indicates the values differed between the two donors at a given time-point. Testis tissue from donor calf No. 1 was stored overnight at 4°C while that of donor calf No. 2 was collected and grafted on the same day. Testis tissue fragments from both donors were grafted into the same recipient mice. [§] When more than 1 graft was collected from a mouse at a given time-point, the number of mice is given in parentheses.

Table 5.2 Correlation coefficients between the characteristics of bison testis tissue xenografts and time after grafting.

	Time (after grafting)			
	Bison calf No. 1		Bison calf No. 2	
	r	P	*r*	P
Graft weight (mg)	0.4	0.03	0.4	0.1
Tubular diameter (μm)	0.6	0.003	0.6	0.002
Tubular density (/mm^2)	-0.6	0.001	-0.5	0.005
Gonocytes/spermatogonia (% of tubules)	-0.4	0.1	-0.2	0.4
Spermatocytes (% of tubules)	0.3	0.2	0.1	0.5
Sertoli-cell-only (% of tubules)	-0.2	0.3	-0.2	0.4
Degenerated (% of tubules)	0.1	0.7	0.2	0.3

Testis tissue from donor calf No. 1 was stored overnight at 4°C while that of donor calf No. 2 was collected and grafted on the same day. Testis tissue fragments from both donors were grafted into the same recipient mice. Correlations were not calculated for end-points with limited data such as those of the later stages of spermatogenesis.

5.4.1.1.2 Tubular density

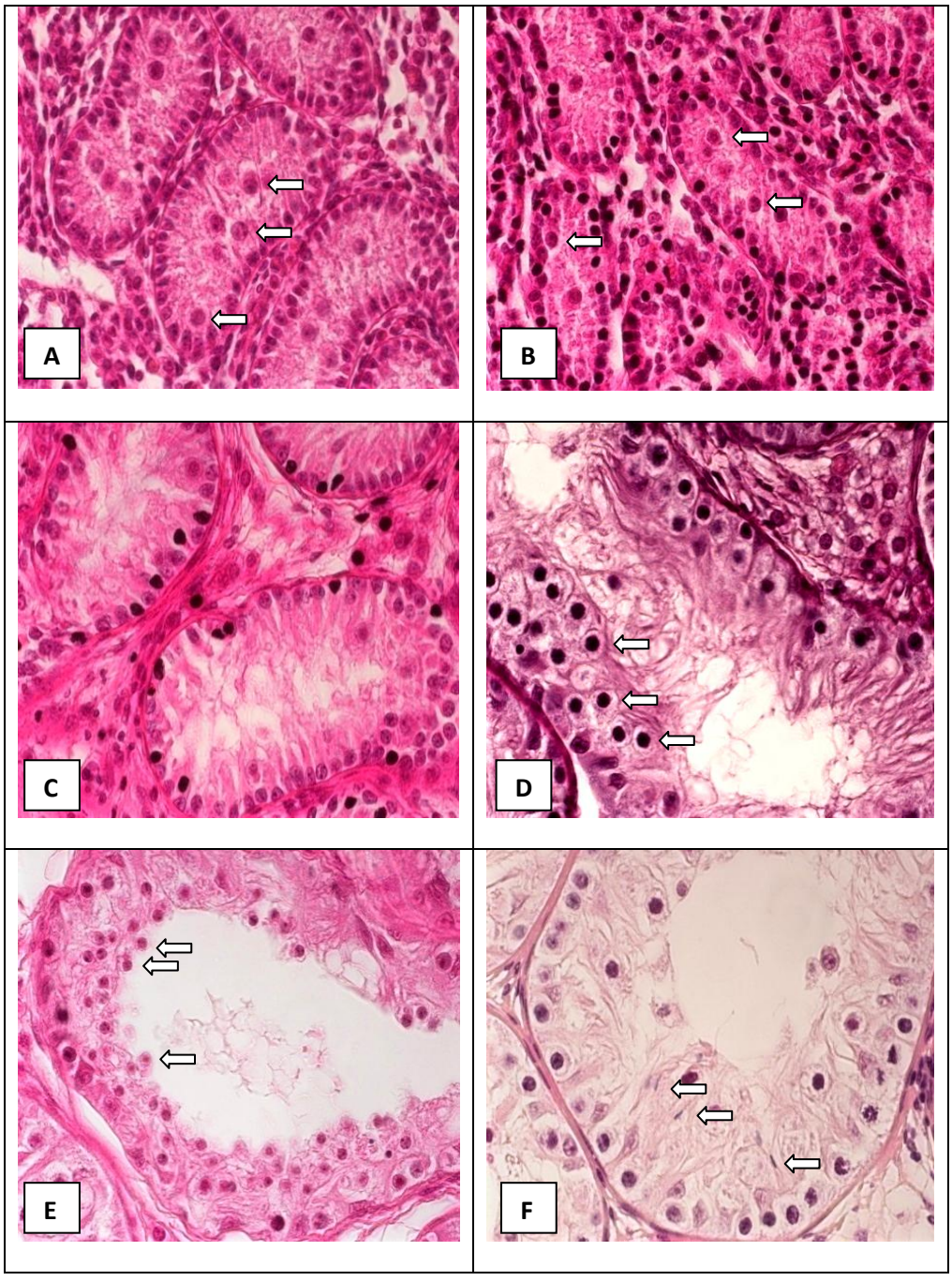
At the time of grafting, the seminiferous tubular density in the neonatal donor bison testis tissues from both donor calves was on average 114 ± 1.5 cords per mm^2. The tubular density in the grafts recovered from recipient mice differed among time-point groups (P = 0.001). As a result of expansion of interstitial tissue, this density was negatively correlated with post-grafting time-points in grafts from both bison calves (r = -0.6 and -0.5, for donor calf No. 1 and 2, respectively, P < 0.001, Table 5.2). For donor calf No. 1, for instance, this density was reduced from ~114 at grafting to ~45 per mm^2 at 2 mo post-grafting and further reduced to ~22 tubules per mm^2 at 14 mo post-grafting (P < 0.05, Table 5.1).

5.4.1.1.3 Tubular morphology

At the time of grafting, the donor testis tissue from both donor bison calves appeared similar and contained seminiferous cords with somatic Sertoli cells, gonocytes (as the only type of germ cells present), and interstitial tissue with Leydig cells (Fig. 5.1a, 1b).

Testis tissue xenografts retrieved at different time-points after grafting displayed slow but gradual development up to elongated spermatids.

Fig. 5.1 Histological appearance of the donor bison testis tissue at the time of grafting and of xenografts recovered from recipient mice at different time-points after grafting.



Photomicrographs from the testis tissue of neonatal bison donors No. 1 (cooled overnight before grafting) and No. 2 (used fresh) at the time of grafting (A and B, respectively, with gonocytes), and those of testis tissue xenografts recovered from recipient mice at 2 mo (C, first tubular expansion), 6 mo (D, first spermatocytes), 12 mo (E, first round spermatids), and 16 mo post-grafting (F, first elongated spermatids). Arrows in each figure point to a few of the most advanced germ cell type present. Scale bar = 100 µm.

There is no consensus as to the terminology of gonocytes that have just migrated from the center of the cord to the basal membrane, and especially the time at which gonocytes attain biological capability as SSCs is not clear (Yoshida *et al.* 2006; McLean *et al.* 2003; Goel *et al.* 2007; Hughes and Varley 1980; Paniagua and Nistal 1984; Orwig *et al.* 2002). The percentages of tubules with gonocytes and those with spermatogonia were recorded as a combined category. There was an overall effect of time ($P = 0.01$) for the percentage of seminiferous cords/tubules in the xenografts containing gonocytes/spermatogonia (as the only type of germ cells present); however, no single time-point group was different from others ($P > 0.05$). At the first time-point (2 mo post-grafting), ~72% of the seminiferous cords did not contain obvious germ cells and were classified as Sertoli-cell-only, while ~29% contained gonocytes (Table 5.1). The relative percentage of tubules with gonocytes decreased over time while that of spermatogonia (as the only type of germ cells present) increased; however, gonocyte-containing cords were observed even in the grafts collected at the last few time-points.

Tubular size expansion in the form of vacuole formation (also reflected in the increased tubular diameter, Table 5.1) started as early as 2 mo post-grafting (Fig. 5.1c) and continued at variable rates due to formation of a complete lumen and multiple germ cell layers throughout the sampling period.

The percentage of graft tubular cross-sections with spermatocytes (as the most advanced types of germ cell present, Fig. 5.1d) differed based on the donor calf and time ($P = 0.04$ and 0.04, respectively); however, no single time-point was different from other time-

points ($P > 0.05$). At 16 mo post-grafting, percentages of graft tubular cross-sections with spermatocytes were significantly higher in the grafts from donor calf No. 1 than from donor calf No. 2 (80% *vs.* 20%, respectively, $P < 0.05$, Table 5.1).

Round and elongated spermatids (as the most advanced germ cell types) were first observed in the seminiferous tubule cross-sections in xenografts collected at 12 and 16 mo post-grafting, respectively (Table 5.1, Fig. 5.1e, 5.1f).

The percentage of graft tubular cross-sections containing Sertoli-cell-only tended to change based on time ($P = 0.05$, Table 5.1). The percentage of degenerated seminiferous tubules in xenografts varied with donor calf ($P < 0.05$) and time. For example, the grafts from donor calf No. 2 had more degenerated tubules at 14 mo post-grafting (0.3%) than at 2 mo post-grafting (39%), or from donor calf No. 1 (2%, $P < 0.05$, Table 5.1).

5.4.2 White-tailed deer testis tissue xenografts

The results obtained from TTX using the white-tailed deer donor are summarised in Table 3. Out of seven recipient mice grafted with deer testis tissue fragments, one mouse died prior to any sampling and was thus excluded from data analysis. From the remaining six mice, a total of 30 out of 48 visible grafts were recovered (63% graft recovery).

Compared with the initial weight of the testis tissue fragments at the time of grafting (~5 mg), the overall average graft weight differed across time ($P < 0.001$), with heavier

grafts (~42 mg) found at 12 mo post-grafting, as compared with those at 2, 4 or 6 mo post-grafting (~13-15 mg, $P < 0.05$, Table 5.3).

5.4.2.1 Histological evaluation of the recovered testis grafts

5.4.2.1.1 Tubular diameter

At the time of grafting, the immature donor testis tissue had seminiferous cords (Fig. 5.2a) that were 50.1 ± 0.3 µm in diameter. The tubular diameters of the grafts examined at 2 mo intervals post-grafting differed over time ($P = 0.003$), and was positively correlated ($r = 0.5$) with time ($P = 0.03$, Table 5.4). For instance, tubules were larger (~161 mg) in grafts at 12 mo post-grafting than at 2 or 4 mo post-grafting (~94-103 mg, $P < 0.05$, Table 5.3).

5.4.2.1.2 Tubular density

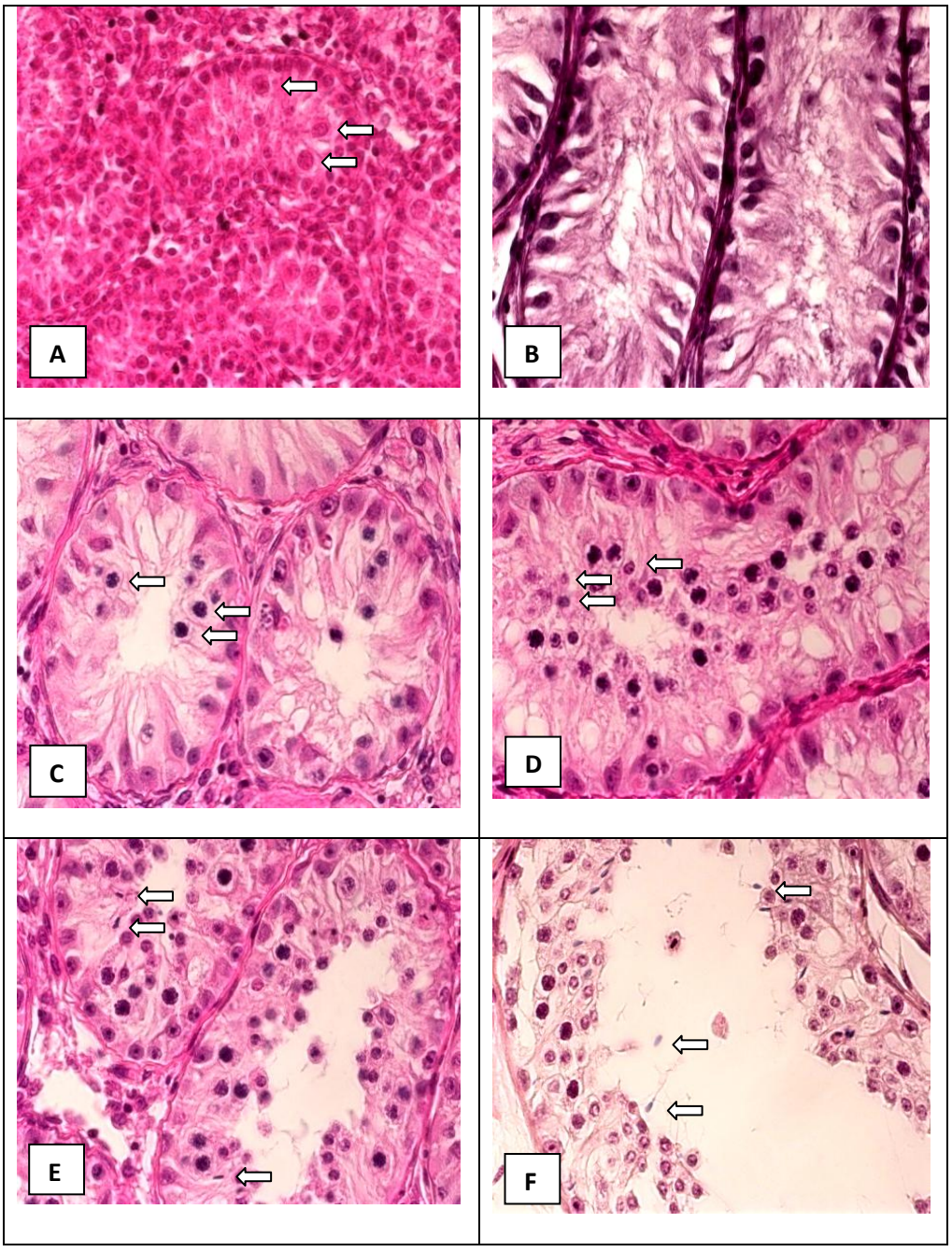
Seminiferous tubular density in the immature deer testis tissue prior to grafting was 79 ± 5.9 cords/mm^2. Tubular density of the grafts recovered from recipient mice had a tendency to differ at different time-points post-grafting ($P = 0.07$, Table 5.3).

Table 5.3 Characterisation of white-tailed deer testis tissue xenografts at different times after grafting.

	Time post-grafting (months)						
	2	4	6	8	10	12	14
No. grafts analysed [§]	3	3	3	3	1	3	14
Average graft weight (mg)	13 ± 0.9[a]	13 ± 3.2[a]	15 ± 1.5[a]	23 ± 2.7	30 ± 0.0	42 ± 7[b]	11 ± 4.2[a]
Tubular diameter (µm)	94 ± 4.5[a]	103 ± 8.5[a]	122 ± 12.9	115 ± 3.4[a]	145 ± 0.0	161 ± 8.0[b]	113 ± 10.0[a]
Tubular density (/mm^2)	21 ± 1.8	25 ± 0.9	21 ± 3.4	22 ± 4	16 ± 0.0	14 ± 1.8	16 ± 1.9
Gonocytes/Spermatogonia (% of tubules)	10 ± 5.4	10 ± 3.0	11 ± 2.9	0.5 ± 0.5	0	16 ± 3	1.9 ± 0.9
Spermatocytes (% of tubules)	0[a]	0[a]	0.3 ± 0.3[a]	18 ± 7.1	7 ± 0.0	27 ± 2.3[b]	7 ± 3.0
Round spermatids (% of tubules)	0	0	0	2 ± 2	0	6 ± 1.3	5 ± 2.8
Elongated spermatids (% of tubules)	0	0	0	0.7 ± 0.7	0	4 ± 3.6	1.8 ± 1.0
Spermatozoa (% of tubules)	0	0	0	0	0	4 ± 3.7	0.2 ± 0.1
Sertoli-cell only (% of tubules)	86 ± 4.7	80 ± 1.9	79 ± 6.8	75 ± 8.6	91 ± 0.0	41 ± 7	58 ± 8.4
Degenerated (% of tubules)	5 ± 2.3	10 ± 4.6	9 ± 5.7	3 ± 1.0	2 ± 0.0	0.5 ± 0.3	26 ± 13.2

Recipient mice were grafted with testis tissue fragments from a 2-mo-old white-tailed fawn, 8 fragments were subcutaneously grafted on the back of each mouse. The xenografts were sampled and evaluated at different time-points after grafting. The average graft weight and histological evaluations were based on the recovered grafts. The average seminiferous tubule diameter and tubular density (the number of the seminiferous tubules/mm^2) were calculated in the largest cross-section of the recovered grafts. For comparison, at the time of grafting, the donor tissue had seminiferous cords that were 50.1 ± 0.3 µm in diameter and at a density of 79 ± 5.9 tubules/mm^2. Data are presented as mean ± SEM. [ab] Values with different superscript letters within each row are significantly different ($P < 0.05$). [§] When more than one graft was collected from a mouse at a given time-point, the number of mice is given in parentheses.

Fig. 5.2 Histological appearance of the donor white-tailed testis tissue at the time of grafting and of xenografts recovered from recipient mice at different time-points after grafting.



Photomicrographs from the immature white-tailed deer donor at the time of grafting (A, with gonocytes), and those of testis tissue xenografts recovered from recipient mice at 2 mo (B, first tubular expansion), 6 (C, first spermatocytes), 8 (D, first round spermatids), 8 (E, first elongated spermatids), and 12 mo post-grafting (F, first spermatozoa). Arrows in each figure point to a few of the most advanced germ cell type present. Scale bar = 100 µm.

5.4.2.1.3 Tubular morphology

The white tailed deer xenografts retrieved at 2, 4, 6, 8, 10, 12 and 14 mo post-grafting displayed gradual development up to complete spermatogenesis (Fig. 5.2). The tubular morphology and the most advanced germ cell types present were recorded for seminiferous tubule cross-sections within grafts and their relative abundance compared among different time-points after grafting.

Tubular expansion was observed in xenografts from as early as 2 mo post-grafting (Fig. 5.2b). The percentage of graft tubular cross-sections with gonocytes/spermatogonia varied among the time-points ($P = 0.02$), but no two time-points differed ($P > 0.05$, Table 5.3).

Spermatocytes (Fig. 5.2c) were first observed at 6 mo post-grafting, and were present in all grafts collected after this time (Table 5.3). The percentage of seminiferous tubules with spermatocytes (as the most advanced germ cell type present) differed among time-points ($P < 0.001$), and increased positively over time ($r = 0.5$, $P = 0.02$, Table 5.4). For example, the highest percentage of tubules with spermatocytes increased (to ~27%) in grafts collected at 12 mo post-grafting, compared with 6 mo after grafting (0.3%, $P < 0.05$, Table 5.3).

Round spermatids (Fig. 5.2d) were first observed in ~2% of tubules in the grafts examined at 8 mo post-grafting and this percentage increased with time ($r = 0.5$, $P = 0.003$, Table 4) and were 5-6% at 12-14 mo post-grafting (Table 5.3).

Elongated spermatids also (Fig 2e) first appeared in grafts in a few tubules (~0.7%) at 8 mo post-grafting, and this percentage was ~4% at 12 mo post-grafting (Table 5.3).

The presence of fully formed spermatozoa (Fig. 5.2f) was first observed within the lumen of ~4% of seminiferous tubules,in grafts retrieved at 12 mo post-grafting and were present in the grafts from the last time-point (14 mo post-grafting, Table 5.3).

Seminiferous tubules containing only Sertoli cells (with no obvious germ cells) comprised the highest percentage of tubules in all categories across all time-points (Table 5.3). Although this percentage varied over time ($P = 0.009$), ranged from ~86% at 2 mo to ~58% at 14 mo post-grafting (Table 5.3), and had a negative correlation with time ($r = -0.7$, $P = 0.002$, Table 5.4), no particular time-point was different from others ($P > 0.05$, Table 5.3).

Although the percentage of degenerated seminiferous tubules in the xenografts did not differ among the time-groups ($P = 0.3$), it ranged from ~5% at 2 mo to ~26% at 16 mo post-grafting ($P > 0.05$, Table 5.3).

Table 5.4 summarizes the correlations between each of the measured outcomes of TTX and the overall effect of time after grafting.

Table 5.4 Correlations between each of the measured outcomes of TTX and the overall effect of time after grafting for white-tailed deer donor.

	Time (after grafting)	
	r	P
Graft weight (mg)	0.3	0.2
Tubular diameter (μm)	0.5	0.03
Tubular density (/mm^2)	-0.6	0.01
Gonocytes/spermatogonia (% of tubules)	-0.2	0.3
Spermatocytes (% of tubules)	0.5	0.02
Round spermatids (% of tubules)	0.6	0.003
Elongated spermatids (% of tubules)	0.4	0.1
Spermatozoa (% of tubules)	0.2	0.3
Sertoli-cell-only (% of tubules)	-0.7	0.002
Degenerated (% of tubules)	0.3	0.2

5.5. Discussion

In the present study, we successfully extended the application of TTX into neonatal/immature bison and deer donors which led to the production of elongated spermatids and spermatozoa from these species in a mouse model. This provides an important first step in achieving gamete production from neonatal/immature donors of rare or endangered ungulates that die prematurely and therefore a new opportunity for conservation of their genetic potential.

In this study, donor testis tissue was collected from two newborn bison calves and one immature white-tailed deer fawn that had died unexpectedly. Since no information was available on the expected time-course of the testicular maturation events in bison or white-tailed deer, after grafting into recipient mice, we surgically removed single sample grafts at 2-mo intervals to monitor the developmental progress of the grafts.

Previous results from TTX showed a wide range of variation depending on the donor species, age or developmental status (Arregui *et al.* 2008b; Oatley *et al.* 2005). We recently found that even donor testis tissue from littermate animals grafted into different recipient mice can show differences in development as grafts (Abrishami *et al.* 2010a). Using the same mouse as recipient for different donors would minimize the potential confounding effects of the host. The two donor bison calves were from the same farm but were genetically unrelated and although their testes arrived at the laboratory at the same time, testes from the first calf had been stored in the refrigerator overnight, while those of the second calf were collected fresh before sending to the laboratory. To control for variation associated with individual donor tissue, we grafted each recipient mouse

with testis tissue fragments from both donor calves (4 testis tissue fragments from each calf on each side).

Although the general pattern of development was similar between the two donor calves, the tissue from donor calf No. 1, that was cooled overnight prior to grafting, showed better developmental potential in some end-points. Since the two donors were of the same age and developmental status, the observed dissimilarity could be related to individual differences or differences in storage conditions after collection. In a previous study, we demonstrated that although the cell viability, graft survival, and developmental competence of porcine tissues cooled prior to grafting were generally comparable to those of fresh testis tissue, a higher percentage of seminiferous tubules with advanced germ cell types could be expected from the tissue cooled for a longer (up to 3 days) period of time (Abrishami *et al.* 2010b). Therefore, aside from the potential individual differences, if indeed the developmental potential of bison testis tissue was positively impacted by low but stable metabolism prior to grafting, we speculate that this beneficial effect could be due to the tissue acclimation to the hypoxic conditions that probably exist immediately after xenografting, and therefore improve subsequent development.

The recovery rates of both bison and deer testis tissue grafts (69% and 63%, respectively) were higher than that reported in the first study of TTX (Honaramooz *et al.* 2002) but lower than those achieved after TTX using porcine donors in more recent studies (Abrishami *et al.* 2010b; Zeng *et al.* 2009). Nevertheless, graft recovery rates

from these new species are promising, given that the donor tissue used for TTX from both species had been collected post-mortem.

The present study provided a first indication of testicular maturation and the timing and the morphology of spermatogenic stages in the grafts from donor bison and deer testis tissue. The tissue grafts from both donor species showed gradual physical development and maturational changes, followed by establishment and continuation of spermatogenesis. The most advanced germ cell type seen in bison and deer testis tissue xenografts were elongated spermatids and spermatozoa which appeared at 16- and 12 mo post-grafting, respectively. There is a lack of comparable information on testicular development and timing of spermatogenic events in these species *in situ*. An observational study on bison living under natural conditions suggested that bison bulls are capable of successfully breeding at 26 mo of age (Berger and Cunningham 1994). It was later shown that bison bulls can reach puberty at 16.5 ± 2.5 mo and commercial bison production systems start using 2-yr-old bulls for natural breeding. Although, the exact age at which the puberty of bull bison starts, and the duration of these developmental processes are largely unknown, our results show a timing of spermatogenesis which is roughly comparable to age at maturation of the male bison (Helbig *et al.* 2007a, b). Therefore, the results of the present study could be a valuable starting point for further studies into the physiology of testis development and function in these species.

General characteristics of testis tissue development from bison in this study resembles those of bovine donors where a similarly large number of tubules lacked differentiated

germ cells at all time-points, but in bison grafts, the process was further delayed by several months. The low efficiency of complete spermatogenesis in bison testis tissue xenografts may be due to an initial loss of germ cells from the donor tissue after grafting and/or impaired meiotic and post-meiotic differentiation, similar to observations in bovine and banteng testis tissue xenografts (Rathi *et al.* 2005, Honaramooz *et al.* 2005).

This study, may pave the way for application of this technique in conservation of closely-related endangered ungulates. Intensive agriculture, environmental pollution, disappearance of natural habitats, and over-hunting are some of the factors that increasingly threaten biodiversity. A number of ungulates are listed as threatened, endangered or extinct. As a general rule, species or breeds with populations below 1000 individuals are considered endangered. Unless effective conservation strategies are introduced to improve or maintain the population size and its limited genetic diversity, extinction may follow (Cseh and Solti 2000). Combination of newly-developed strategies including the preservation of testis tissue prior to grafting (Abrishami *et al.* 2010b; Yang and Honaramooz 2010a; Yang *et al.* 2010b), the use of TTX, and the storage of spermatozoa after retrieval, will allow the future use of the spermatozoa in ICSI (Honaramooz *et al.* 2002; 2004; 2008) which can potentially lead to production of progeny (Schlatt *et al.* 2003; Nakai *et al.* 2010).

In addition, our results of TTX from immature donor bison calves may be used for more direct applications in the management of wild bison population. The difficulty of balancing between the need for wildlife conservation and control of serious zoonotic diseases can be exemplified in the case of tuberculosis and brucellosis in the wild bison.

In Canada, efforts to eradicate bovine tuberculosis in cattle started more than 110 years ago (Kaneene and Pfeiffer 2006) and have generally been successful in eliminating it from cattle herds. However, infected or carrier wild bison, especially those in and around certain national parks in Canada remain the only major impediment in completing this important task. This is because wild bison are an important maintenance reservoir of *Mycobacterium bovis*, and therefore are considered its only wildlife reservoir in Canada. In the last 50 years, multiple attempts have been made to recover disease-free bison from affected national parks but none have been successful (Tessaro 1986; Wobeser 2009).

The policy of whole-herd depopulation was applied for confirmed cases of tuberculosis in cattle herds after tuberculosis was almost eliminated. The same approach has been proposed for the wild bison but the lack of a plan to preserve genetic diversity made this approach unacceptable. Until a proper solution is found, there will be an enormous biosecurity risk for other wild bison herds, currently thought to be disease-free, creating a much larger conservation dilemma and a geographically larger risk to livestock. Therefore, innovative strategies that can both conserve the genetic diversity of the wild ungulates and at the same time eliminate the health risk are desperately needed. This includes the use of novel methods for preservation of gametes from large numbers of individuals within the existing population of wild bison, and the development of protocols for successful banking of the bison semen is a good example (Lessard *et al.* 2009; Pérez-Garnelo *et al.* 2006). In gamete banking, mature males provide an abundant source of gamest because epididymal spermatozoa can be recovered and preserved, even after death. However, conservation of genetic diversity at this scale cannot overlook the

potential contribution that can be made by animals that die prematurely and before spermatozoa are produced.

Following TTX from immature donor white-tailed deer, the seminiferous tubules developed gradually until complete spermatogenesis was observed. The first spermatocytes appeared at 6 mo post-grafting, followed by round spermatids as well as elongated spermatids at 8 mo post-grafting, and spermatozoa at 12 mo post-grafting (or 14 mo of age for the tissue because the donor was 2 mo old at grafting). Comparison of this timing with that *in situ* is difficult because no documented information is available on testicular growth and development. Deer are seasonal breeders and it is generally believed that white-tailed deer are fertile in the autumn following their year of birth (i.e., ~18 mo of age), although some buck fawns are believed to be sexually precocious and able to impregnate does even in their first year (i.e., at ~6-7 mo of age) (Rue 1997). Future experiments may explore the possibility, although unlikely (Schlatt *et al.* 2002; Wistuba *et al.* 2004), of an inherent mechanism for seasonal response of the donor tissue from a seasonal species (e.g., deer) developing as grafts in a non-seasonal recipient (mouse), and also of seasonal cues on timing of testicular maturation in such grafts. The results of our study can also have implications for farmed white-tailed deer operations where promising male fawns from prized genetic background may die prematurely.

In conclusion, the results of TTX from neonatal/immature donor bison and white-tailed deer are promising and may be expandable to closely-related rare or endangered wild ungulates.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSIONS AND FUTURE APPLICATIONS

6.1 General discussion and conclusions

The materials presented in this thesis are the result of experiments to elucidate a number of factors affecting the outcome of testis tissue xenografting (TTX) (Chapters 3 and 4), and on preservation of male gonadal tissue from immature specialised livestock (Chapter 5). Specifically, we evaluated the effects of : 1) different recipient-related factors and 2) different numbers of initial donor testis tissue fragments on the outcome of TTX; and 3) extended the application of TTX to bison and white-tailed deer donors, as a model for genetic conservation of rare or endangered ungulates.

Recently, TTX was introduced as an approach to maintain the structural integrity and cell associations of the donor testis tissue, allowing it to undergo testicular maturation and produce spermatozoa from donors of several species in a laboratory mouse (Honaramooz *et al.* 2002). This strategy provides for powerful and unique applications including its use as an option for the study and manipulation of spermatogenesis and for the preservation of male germlines. However, the extent of the observed spermatogenesis in testis grafts dramatically differed in different TTX studies, depending on the choice of donors and recipients. The majority of these studies followed the procedures described in the first report in which castrated male nude mice were used as recipients and in which 8 fragments of donor testis tissue were grafted under the skin

of each recipient mouse (Honaramooz *et al.* 2002; Rodriguez-Sosa and Dobrinski 2009). Therefore, there is great value in re-examining the methodology of TTX to optimise its efficiency and consistency of outcome. So far, there are no reports of TTX studies that have systematically investigated the effects of factors related to recipient mice or the number of tissue fragments used.

We aseptically castrated neonatal piglets at the University of Saskatchewan's Prairie Swine Center as a source of donor testis tissue for the first two experiments (Chapters 3 and 4). From previous studies in our laboratory, we knew that the testes collected from this genetically-defined herd would provide a homogenous supply of donor tissue, both in terms germ cell proportion (Yang *et al.* 2010c) and developmental potential of the tissue (Abrishami *et al.* 2010b). From previous TTX studies, we also knew that we could anticipate a high graft recovery rate and complete spermatogenesis by 8 mo post-grafting (Honaramooz *et al.* 2002; 2008; Abrishami *et al.* 2010b). Therefore, the endpoints of this study were chosen to reflect even subtle differences in the outcome of porcine TTX.

For the first study (Chapter 3), we grafted piglet testis tissue into recipient mice using a 2×2×2 factorial design to simultaneously investigate the effects of recipient mouse strain (nude *vs.* SCID), gender (male *vs.* female), and gonadal status (intact *vs.* gonadectomised) on the outcome of TTX. Nude mice were chosen because they are the most commonly used strain of recipients for TTX, and they were compared with SCID mice. Overall results showed that the grafts recovered from SCID mice were ~1.8-fold

heavier and had higher values of spermatogenic development, compared with those of nude mice.

Rathi *et al.* (2005) also used nude and SCID mice for xenografting of bovine testis tissue. They pooled the data from both recipient models because they did detect a difference. Geens *et al.* (2006) compared testicular development after TTX of mouse and human donor tissue to nude and SCID mice. They also reported similar results for both strains of mice. In another study (Watanabe *et al.* 2009), three different types of immunodeficient mice (nude, SCID and NOG) were used as recipients of TTX and germ cell injection. They concluded that the three types of recipients showed the same level of spermatogenesis after TTX, but SCID mice appeared to be a more acceptable recipient model for germ cell injections. They also proposed that the extent of immunodeficiency does not affect testicular tissue development as results in NOG mice did not differ from those of SCID or nude mice. Despite these reports showing no conclusive differences between nude and SCID recipient mice (Rathi *et al.* 2005; 2006; Geens *et al.* 2006; Watanabe *et al.* 2009), our results clearly showed that grafts from SCID mice can be expected to have greater gross and histological development. Results of the previous studies may be explainable by the number of animals used, which may have been insufficient, compared to our study, to show a difference.

Another important recipient-related factor that was evaluated in this experiment (Chapter 3) was the mouse gender. We were interested to establish whether female recipient mice were capable of supporting the development of testis xenografts. We observed an overall ~10% higher graft recovery rate could be achieved in male mice

than in females, and that the grafts from males were ~3.7-fold heavier than from females. Grafts from male recipient mice also had greater development, especially in the form of advanced stages of spermatogenesis. This is the first time that differences in recipient mouse gender have been shown to play a role in the outcome of TTX.

The third recipient-related factor assessed in this experiment (Chapter 3) was the gonadal status of recipient mice. This variable was included because typically recipient mice are castrated prior to TTX, and we were interested to test whether gonadectomy of the recipient mice indeed enhances the results of TTX. The potential benefit of gonadectomy is believed to be related to the compensatory rise in serum levels of gonadotropins which may further promote development in grafts (Honaramooz *et al.* 2002; Schlatt *et al.* 2003; Paris and Schlatt 2007). However, our results showed that gonadectomy of the recipient mouse do not affect the measured outcomes of TTX. Interestingly though, even the intact female recipient mice were fully capable of supporting testicular development and sustaining complete spermatogenesis in the grafts. Since the outcomes of TTX in intact and gonadectomised recipient mice did not differ while those from males and females differed, non-hormonal factors may contribute to gender differences. One such contributing factor could be the mouse body weight, because male recipient mice tended to be heavier than females, and there were positive correlations between the body weights and average graft weights, as well as a few other criteria of graft development.

The second experiment (Chapter 4) was a continuation of our main goal in examining factors that can optimise the results of TTX. In this experiment, we investigated the

effect of changing the initial number of porcine testis fragments used for TTX. We chose castrated male nude mice, because they have been the conventional recipient mouse model for TTX. Therefore, in addition to the conventional number of donor testis fragments (8), we used 2, 4 or 16 fragments (~1 mm^3 or ~5 mg each) per mouse. It should be noted that this experiment (Chapter 4) was conducted at the same time as our first experiment (Chapter 3); therefore, we did not know that male SCID mice would provide superior results compared with nude mice as the traditional recipient model. Our results showed that not only the total graft weight, but also the average graft weights was higher in the group of mice that received 16 testis tissue fragments, compared with the group that received two testis tissue fragments. Therefore, the development of much greater number of testis fragments (i.e., twice the conventional number) can be easily supported by the recipient mice and in fact, graft development can be improved. In a recent study, Rodriguez-Sosa *et al.* (2010) tried to increase the number of seminiferous tubules by grafting two flat strips of ovine testis tissue per recipient mouse. When comparison was made based on per-unit of testis tissue, our results with cube-shaped small testis fragments were up to 27-fold more efficient in physical development than the much larger flat-shaped strips of sheep testis tissue (Rodriguez-Sosa *et al.* 2010). The graft recovery rate and percentage of seminiferous tubules completing spermatogenesis reported Rodriguez-Sosa *et al.* (2010) were similar to two other studies involving ovine TTX (Zeng *et al.* 2006; Arregui *et al.* 2008). The differences in the shapes of the xenografted testis tissue could be a possible explanation for the difference in the rate of physical and histological growth obtained in our study compared with that of Rodriguez-Sosa *et al.* (2010). Previous studies demonstrated that small testis tissue fragments allow for a higher degree of vascularisation after TTX (Schlatt *et al.* 2006b;

Schlatt *et al.* 2010). The histological development of grafts from the group of mice carrying 16 fragments in our study was also more advanced when compared with those of mice with 2 fragments. In addition, the extent of tubular damage was significantly higher in grafts from the group of mice that received two testis fragments. Therefore, we concluded that recipient mice can receive at least 16 fragments per mouse and still provide higher physical and spermatogenic development than that of the conventional 8 fragments. In our analysis, we also measured the vesicular gland indices as indicators of the bioactive androgens produce by the grafted testis tissue (Honaramooz *et al.* 2002; Schlatt *et al.* 2003). The group of mice that received 16 testis tissue fragments had a ~5-fold higher vesicular gland indices than those that received only two fragments (0.5% *vs.* 0.1%, respectively). These indices were in fact closer to those of intact nude mice which have been reported to range from 0.4% to 1.1% (Schlatt *et al.* 2003). These results indicate that testosterone from the porcine grafts was able to maintain the vesicular glands of the castrated recipient mice, although given the mass of the grafted tissue compared to the normal size of mouse testes in intact animals, probably not as efficiently.

Through the collective results from our two experiments (Chapters 3 and 4), we conclude that male SCID mice (which do not need to be castrated), especially if grafted with at least 16 fragments, provide a more suitable TTX recipient model than the conventional model (castrated male nude mice receiving 8 testis tissue fragments). Therefore, we determined and optimised the choice of recipient mice and the number of grafts to improve TTX results, in terms of harvesting a higher mass of testicular grafts with more advanced spermatogenesis. These results are useful for immediate application

in studies utilizing TTX, especially for use with rare or endangered donor species where the amount of donor tissue may be a limiting factor.

Using TTX, complete spermatogenesis in the xenografts from many domestic donor species has been achieved; however, its application in non-domestic ungulates has been limited. One of the important applications of TTX is its use in conservation of valuable, rare or endangered species/breeds by producing spermatozoa from immature donor testes. Therefore, TTX can be a unique solution for the problem of rescuing the genetic material of valuable males that die prematurely (Pukazhenti *et al.* 2006; Arregui *et al.* 2008a). To expand the use of this technique into vulnerable species, especially rare or endangered ungulates, preliminary experiments using closely-related species are warranted.

In our third experiment (Chapter 5), testis tissue from two newborn bison calves and one immature white-tailed deer fawn, that had died unexpectedly, were collected after death and xenografted into gonadectomised nude mice. Since no information was available on the time course of testicular maturation events in bison or white-tailed deer, we surgically removed single sample grafts at 2-mo intervals to monitor the developmental progress of the grafts. The grafts from both donor species showed gradual physical development and maturational changes of the tissue, followed by establishment and continuation of spermatogenesis. The recovery rates of both bison and deer testis tissue grafts (69% and 63%) were higher than in earlier reports of TTX (Honaramooz *et al.* 2002), but lower than those achieved after porcine TTX in our experiments (Chapters 3 and 4), or those of other recent studies (Abrishami *et al.* 2010b; Zeng *et al.* 2009).

Nevertheless, these results are promising given that they constitute the first report from these species and that donor tissue used for TTX from both species had been collected post-mortem.

For bison grafts, the average weight, tubular density and tubular diameter varied across the time-points, and near complete spermatogenesis (appearance of elongated spermatids) was observed at 16-mo post-grafting which is roughly comparable to age at maturation of the male bison (Helbig et al 2007a,b). The absence of differentiated germ cells was evident in some seminiferous tubules at all post-grafting time-points, similar to that encountered with bovine donors, but with a further delay of several months before complete spermatogenesis was observed in bison grafts. The initial loss of germ cells after grafting and/or impaired meiotic and post-meiotic differentiation could be a possible mechanism for the low efficiency of complete spermatogenesis in bison xenografts, similar to what has been observed in closely-related species (Rathi et al. 2005; Honaramooz et al. 2005).

Following TTX from donor deer, the average graft weight and tubular density differed across the time-points and the seminiferous tubules developed gradually until 12 mo post-grafting (or 14 mo of age for the tissue since the donor animal was 2 mo old at the time of grafting) when spermatozoa were observed. Comparison of this timing with that of *in situ* is difficult because deer species are seasonal breeders. It is generally believed that white-tailed deer are fertile in the autumn following the year of birth (i.e., ~18 mo of age). However, some buck fawns are able to impregnate does even in their first year of life (i.e., at ~6-7 mo of age) (Rue 1997). In future TTX experiments, it would be

interesting to explore whether seasonality plays a role in timing of testicular maturational changes in grafts from seasonal-breeder species developing in a non-seasonal-breeder recipient (mouse).

Through multiple experiments in this thesis, we successfully improved the outcome of TTX (Chapters 3 and 4) and successfully extended the application of TTX into bison and deer as models for rare or endangered ungulates (Chapter 5). Based on the cumulative conclusions made in this thesis (Chapters 3, 4 and 5), we expect that TTX from immature rare or endangered ungulates could also be successful in achieving complete spermatogenesis and production of fertile spermatozoa, especially if applied using the superior recipient mouse model (male SCID recipient mice) carrying at least 16 testis tissue fragments. This will provide new opportunities for conservation of genetic potential from rare or endangered ungulates that die prematurely.

6.2 Future applications and research directions

The results of these studies will likely have immediate and future applications. The result of Chapters 3 and 4 will help researchers involved in the study and manipulation of testis function to choose a more acceptable recipient model and appropriate number of grafts, and those of Chapter 5 may help wildlife conservation researchers to use an *in vivo* model for maturation of testicular tissue from important non-domestic species.

Through systematic studies, we were able to improve the outcome of TTX by optimising some of the recipient and donor factors. However, there is still room for improvement and a number of factors remain to be studied. One such factor that could potentially

affect the consistency of TTX outcome, in terms of physical growth and histological development, is neo-vascularisation after grafting. Insufficient angiogenesis may be an important factor affecting further growth of the newly grafted testis tissue. Future studies could examine whether limited neo-vascularisation is indeed a problem for some grafts and if so to what extent. Molecular effects on vascularisation could then be explored and ways to improve vascularisation tested to improve the TTX outcome.

A number of animals including ungulates are listed as threatened, endangered or extinct. Wildlife laws have been established to improve or maintain the population size and to protect animals from extinction. Enforcement of these laws, along with prevention of illegal wildlife trade and providing environmental education have all been partially successful in animal conservation and protecting natural habitat. One of the valuable tools in assessing the risk factors and finding solutions for preservation of endangered animals is research, especially in the field of gonadal, germ cell and tissue preservation. A combination of these types of studies with other assisted reproductive technologies such as embryo transfer, *in vitro* fertilisation and ICSI could be a potential solution for some of the wildlife conservation dilemmas.

In wild bison, other considerations such as the health and economy of Canada are forcing authorities to come up with more creative ways of balancing the need for conservation of wildlife and disease control. A large number of wild bison in certain national parks are carriers of tuberculosis and brucellosis, but unlike cattle, whole-herd depopulation of wild bison herds is not an option. Therefore, a plan to preserve genetic diversity would require novel techniques that can preserve the genetic diversity of most

if not all animals within a herd including immature animals. Results of our study suggest that TTX may be a valuable strategy to preserve the male germline of rare or endangered animals that die before puberty. Future studies could seek to improve TTX outcome form donor bison and other wild ungulates using optimization of the recipient. Other future studies could fine-tune testis tissue cryopreservation protocols developed earlier in our laboratory (Abrishami *et al.* 2010b) to be applied for donor specialised livestock to ensure the availability of testis tissue in cases where facilities or recipient mice are not available at the time of unexpected deaths. Examination of fertilization competence of bison sperm obtained from testis tissue xenografts would further confirm suitability of TTX for such application. Our results have therefore provided an important starting point in applying TTX for immature males of rare or endangered species to help maintain genetic diversity.

Another application of our studies is to help research on spermatogenesis and testis development. This, for instance, could be in the form of using genetically-modified isolated testis cells in combination with non-transgenic testis cells or with other cells of species for a co-grafting transplantation. The results from experiments using such models could explain the unknown roles of specific genes in testis development and spermatogenesis and elucidate the cell-to-cell interactions during spermatogenesis.

One of the current limitations of TTX is poor outcome of development after grafting of mature donor testis tissue. To overcome this limitation and look for the options that can take advantage of the resources provided by older donors, research into the exact reasons for failure in adult testis tissue xenografting seems necessary.

To overcome some of the infertility concerns in boys undergoing cancer treatment, the study of cryopreservation and xenografting of testis tissue might be one of the best options. However, the potential risks of human TTX such as disease transmission create some ethical issues for these types of studies and need to be explored.

For investigating the mechanism involved in the cytotoxic effects of cancer therapy, TTX using our new model can provide a tool for investigation.

Given the high rate of species variety in the outcome of TTX, the use of other wild ungulate species using our refined recipient model will provide more definite evidence for the application of TTX for endangered ungulates.

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