

STEM CELL-BASED TECHNOLOGIES OF ASSISTED REPRODUCTION
IN THE CAT AND DOG

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STEM CELL-BASED TECHNOLOGIES OF ASSISTED REPRODUCTION IN THE CAT AND DOG

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Spermatogonial stem cells (SSC) exist on the basement membrane of the seminiferous tubules in the testis throughout a male animal's life. These cells self-renew to maintain a stem cell population while producing daughter cells that differentiate into sperm. Because of their relatively undistinguishable morphology, very little was known about them until testis xenografting and spermatogonial stem cell transplantation (SSCT) were developed. Both technologies are promising tools to preserve male genetic information as well as to study the characteristics of SSC. In this dissertation, I investigate testis xenografting and SSCT in cats and dogs, which are important animal models for biomedical research.

First, I examine the effect of donor age on spermatogenesis in feline xenografts and the ability of xenograft-derived sperm to support embryo development. In addition, I compare the outcomes of xenografting using testis tissue from two additional species of carnivore, dogs and ferrets. My studies indicate that the optimal donor age was just prior to onset of puberty and xenograft sperm had poor fertility compared to control sperm, necessitating further investigation. Furthermore, the comparative study demonstrates differences in the ability of testicular tissue from different species of carnivore

to establish spermatogenesis following testicular xenografting. Second, I describe my development of all three major steps of SSCT in the cat and dog including depleting endogenous male germ cells in recipients, isolating male germ cells from donors and injecting the isolated germ cells. My studies provide a foundation to perform SSCT in the cat, and I was successful at performing SSCT in the dog. Finally, I examine multiple steps to develop transgenesis in dogs, which will enhance their utility as genetic models. This study includes 1) methodology for the accurate staging of dog blastocysts, 2) attempted derivation of canine embryonic stem cells (cESC), 3) manipulation of blastocysts, and 4) transfer of canine embryos. Our results demonstrate that canine transgenesis mediated via cESC and/or SSCT are technically feasible.

These studies examine the potential of stem cell-based technologies in the preservation of male genetic information and biomedical research. In addition, my work provides a foundation to achieve transgenesis in the dog.

BIOGRAPHICAL SKETCH

Yeun Hee Kim was born in Seoul, Korea in 1978. Despite only seeing building projects and paved roads in childhood neighborhood, which has become one of the busiest places in Seoul, her natural instinct always drew her to nature. Therefore, as she completed her B.S in life sciences at the University of Seoul in 2001, she joined Dr. Hang Lee as an MS/Ph.D student in conservation genetics at Seoul National University (SNU). There, she studied the genetic and reproductive profiles of Asiatic black bears, which are almost extinct in South Korea. She also participated in a reintroduction program of the bears into the wild in South Korea during her graduate study. After two years at SNU, she got a scholarship for one-year training program from the Brain Korea 21 project to study at Cornell University. On March 17th 2003, she landed in the United States and came to Cornell University. For the first 6 months, she worked with Dr. George Kollias in zoo and wildlife medicine and then joined the laboratory of Dr. Alexander Travis at the Baker Institute for Animal Health, based on a shared interest in wildlife conservation. As she loved her projects very much and developed a strong interest in bench work over fieldwork, she decided to apply to the graduate program at Cornell University. She was admitted to the program and started her doctoral training anew in 2005. Upon completion of her Ph.D, Yeun Hee Kim will join her husband in New Haven, Connecticut, where she will pursue postdoctoral training.

Dedicated to my parents

Sunkyu Choi
Mookeun Kim

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CHAPTER 1

Introduction and Literature Review

Spermatogenesis is a complex process involving hormonal regulation of, and communication between, various somatic cells and germ cells, to achieve its ultimate goal of producing haploid male gametes. Although a male animal doesn't produce sperm until the animal undergoes puberty and becomes sexually mature, spermatogonial stem cells (SSC), the stem cells of the male germ line, reside and proliferate in the testes beginning soon after the birth of the animal. Although, these stem cells were identified several decades ago, very little has been discovered about SSC. Their largely unremarkable morphological characteristics have hindered research. Above all, the absence of a single assay to characterize the cells was the greatest obstacle for this field. However, two novel technologies were introduced within the last two decades, spermatogonial stem cell transplantation in 1994 and testis xenografting in 2002, and these have played critical roles in accelerating SSC research. Both techniques allow us to remove male germline stem cells from an original animal and transfer them to host animals without losing the stem cell nature of the transplanted cells. In this chapter, I intend to review how male germ cells develop from early embryos and what events are involved in sperm production. I shall then review the properties of spermatogonial stem cells, and the technologies based upon them. Because my research has been focused on the cat and dog, I shall close with a review of the potential of these species as biomedical research models.

Male germ cell development

1. Fetal germ cell development

Mammalian germ cells are induced to form at the posterior region of the epiblast, at the junction of the extra-embryonic ectoderm, epiblast, primitive

streak, and allantois (Figure 1.1.a) (Hogan 2002). Among the many cells in the area, only certain cells will develop to become primordial germ cells (PGC). At embryonic day 6-6.5 in rodents, the precursors of PGC express BMP4 and BMP8b (Figure 1.1.a) (Lawson, Dunn *et al.* 1999; Ying, Liu *et al.* 2000). Around embryonic day 7, this cluster of cells emerges from the posterior primitive streak, and a few cells in the center of the cluster expressing both *fragilis* and *stella*, become committed to the germ cell fate (Figure 1.1.b) (Saitou, Barton *et al.* 2002). Mammalian PGC migrate directly into the endoderm from the posterior region of the primitive streak (Figure 1.1.c). Although the cells exit the hindgut on day 9, PGC are seen migrating into the genital ridges during the following day. By embryonic day 11.5, the PGC enter the developing gonads (Anderson, Copeland *et al.* 2000; Molyneaux, Stallock *et al.* 2001). Although PGC express germline-specific genes (Yabuta, Kurimoto *et al.* 2006), they are still pluripotent and are capable of producing differentiated cell types representing all three germ layers (Seydoux and Braun 2006). During their migration to the genital ridges, PGCs proliferate from 10-100 cells to 2500-5000 cells by day 12. In females, PGCs form oocytes, which stop dividing mitotically and enter meiosis, whereas in males the cells become gonocytes in the sex cords (Goossens and Tournaye 2006).

Because PGC still retain pluripotency and can form teratomas *in vivo* (Noguchi and Stevens 1982; Regenass, Friedrich *et al.* 1982) and pluripotent stem cells *in vitro* (Matsui, Zsebo *et al.* 1992; Resnick, Bixler *et al.* 1992), gonocytes can be considered to be the first stem cells that are committed to the male germ cell lineage. In rodents, these gonocytes become arrested in the G₀/G₁ phase of the cell cycle after a few days of proliferation (Vergouwen, S.G. *et al.* 1991). Shortly after birth, the gonocytes are released

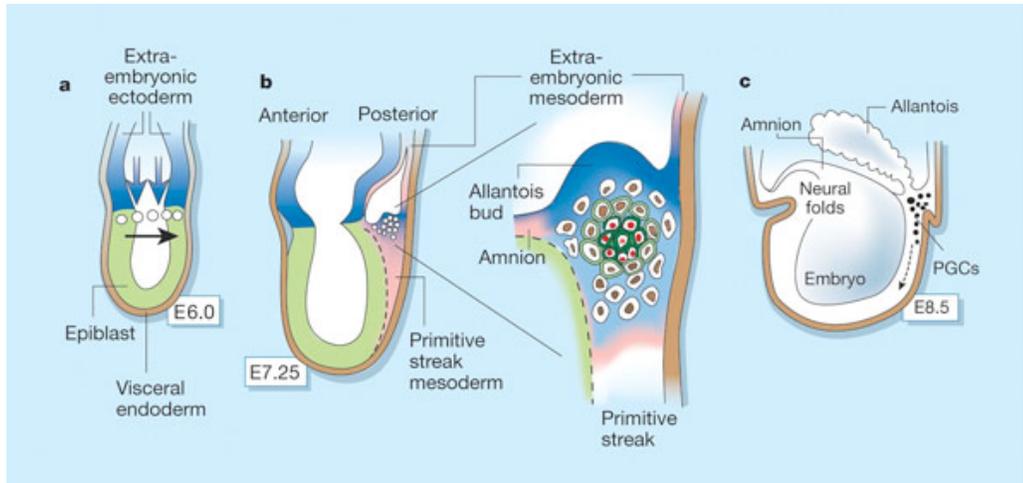


Figure 1.1. Model for the development of mammalian primordial germ cells. a) BMP signals (blue) from extra-embryonic ectoderm induce neighboring epiblast cells (open circles) to become precursors of PGC and extra-embryonic mesoderm. These cells move (black arrow) toward the posterior epiblast during gastrulation. b) The cells emerge from the posterior primitive streak on embryo day 7. A small cluster of cells in the center, expressing both fragilis (green) and stella (red), will become PGCs. c) Once PGCs are specified, they migrate to the future gonad (dashed arrow). Reprinted with permission from Macmillan Publishers Ltd: Nature (418, pp.282), copyright (2002).

from arrest and become SSC on the basement membranes of the seminiferous tubules (Bellve, Cavicchia *et al.* 1977; Vergouwen, S.G. *et al.* 1991). However, it was reported that there are heterogeneous populations of these cells in the neonatal testes including a small population of cells that become stem cells (de Rooij 1998) and a significant number of cells that eventually degenerate (Wang, Nakane *et al.* 1998). Based on their distinct morphological characteristics, Orwig *et al.* (Orwig, Ryu *et al.* 2002) identified the two subpopulations of gonocytes, pseudopod and round, from rat neonatal testes and showed that pseudopod cells become stem cells whereas round cells undergo apoptosis. Apoptosis is involved in all stages of male germ cell development and its role in balancing survival and death signals is critical to prevent spermatogenic dysfunction (Jahnukainen, Chrysis *et al.* 2004; Russell, Chiarini-Garcia *et al.* 2002). Therefore, apoptotic activity of round gonocytes, which may represent the cells that failed to migrate to the basement membrane of the seminiferous cords, might be critical to establish normal spermatogenic development in the early testis.

2. Spermatogenesis

Spermatogenesis is the process in which spermatogonia produce the mature male gametes, spermatozoa, within the seminiferous tubules. This process includes a series of mitotic divisions of spermatogonia, two meiotic divisions of spermatocytes, extensive morphological transformation of the spermatids, and the release of the maturing spermatozoa into the lumen of the tubules. In this section, each phase of germ cell development will be reviewed.

2.1. Mitotic phase of spermatogenic cells

Once gonocytes migrate from the center of the seminiferous cords to the basement membrane of the cords, they transform into spermatogonial stem cells (SSC) within postnatal day 6 in the mouse (McLean, Friel *et al.* 2003). During the proliferative phase, spermatogonial stem cells undergo mitotic divisions to produce both new stem cells and daughter cells that are committed to differentiation into spermatozoa. The most primitive spermatogonia are called 'type A spermatogonia' and have a minimum level of heterochromatin in the nucleus. With successive increases in the level of heterochromatin, the type A spermatogonia become intermediate spermatogonia (In), which in turn become type B spermatogonia, which display significant heterochromatin in the nucleus. The type A spermatogonia consist of subtypes of cells, types A-single (A_s), A-paired (A_{pr}), A-aligned from 4 to 16 cells (A_{al}), and A_1 , A_2 , A_3 and A_4 (de Rooij and Russell 2000). A_s cells are considered to be the true spermatogonial stem cells (SSC) which will divide into either two A_s cells or a pair of A_{pr} . A_{pr} cells divide into chains of A_{al} cells, but they are still connected through intercellular bridges (Figure 1.2) (Weber and Russell 1987).

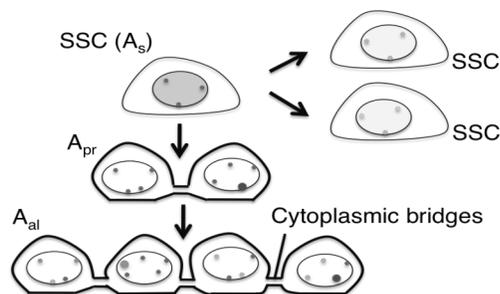


Figure 1.2. The division and proliferation of undifferentiated spermatogonial cells. SSC: spermatogonial stem cell, A_s : type A-single spermatogonia, A_{pr} : A-paired spermatogonia, A_{al} : A-aligned spermatogonia.

A_s , A_{pr} , and A_{al} are considered to be undifferentiated spermatogonia, and they share similar phenotypes and likely also share molecular characteristics (de Rooij 2001; de Rooij and Russell 2000). A recent study showed that A_{pr} and A_{al} spermatogonia can also resume self-renewal in the condition of loss of actual stem cells (Figure 1.3) (Nakagawa, Nabeshima *et al.* 2007). A_{al} cells progress to become A_1 cells without a mitotic division. A_1 cells then divide and differentiate into A_2 , A_3 , A_4 , In and B spermatogonia. It is challenging to distinguish between the undifferentiated spermatogonia by biochemical or molecular characteristics. However, it has been shown that in mice these cells are distinguishable from differentiating spermatogonia, such as A_{1-4} , In and B spermatogonia, based on differential expression of several cell surface markers including c-kit (Schrans-Stassen, van de Kant *et al.* 1999), β_1/α_6 -integrin (Shinohara, Avarbock *et al.* 1999) and Thy-1 (Kubota, Avarbock *et al.* 2004). The undifferentiated spermatogonia do not express c-kit but β_1/α_6 -integrin and Thy-1 whereas the differentiated spermatogonia have the opposite expression pattern. In mice, this differential expression of cell surface markers can be used to investigate the different properties between undifferentiated and differentiated spermatogonia. Additional markers for murine male germ cells at different stages of development are shown in Table 1.1.

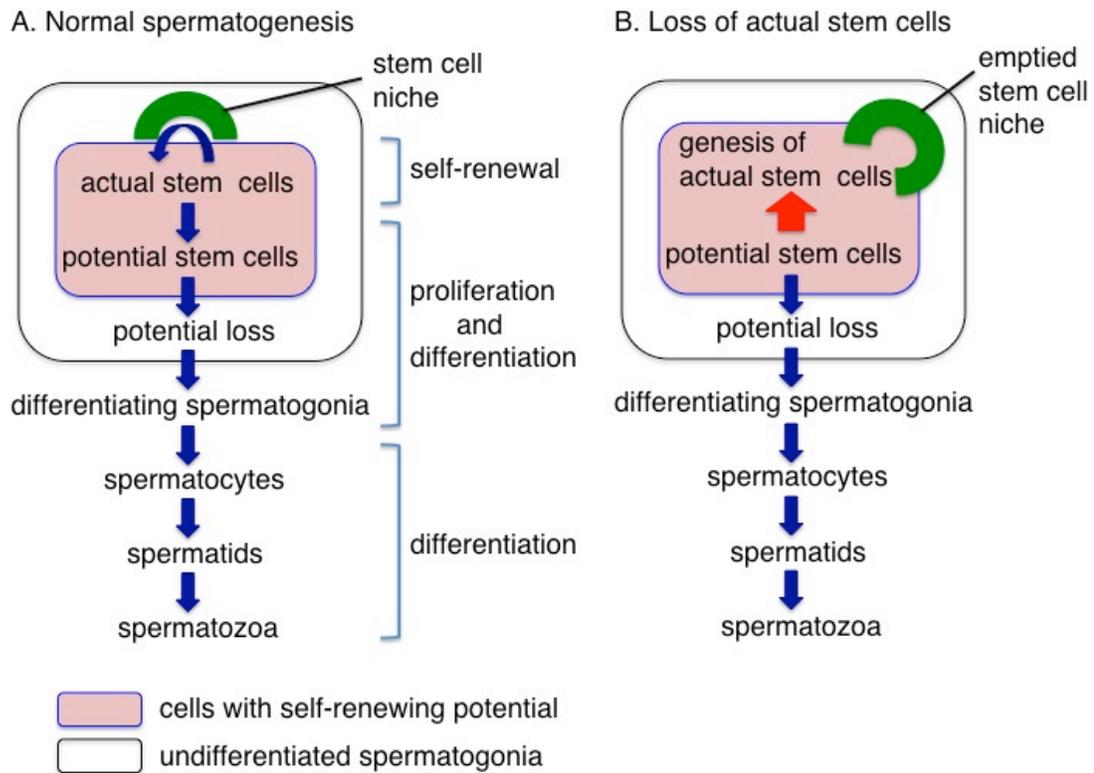


Figure 1.3. Model of the spermatogonial stem cell compartment. A. During a normal cycle of spermatogenesis, stem cell potential is not limited to the cells that actually self-renew in the stem cell niche (actual stem cells) but also some cells that do not normally self-renew might possess this potential (potential stem cells). Both of these compartments are included in the undifferentiated spermatogonia entity. B. Upon loss of the actual stem cells, the potential stem cells would switch their mode from proliferation to self-renewal, generating a pool of new actual stem cells (indicated by a red arrow). According to Nakagawa et al., 2007. (Nakagawa, Nabeshima *et al.* 2007)

Table 1.1. Description of differential expression during male germ cell development in mice

Markers	PGCs	gonocytes	SSC	Differentiated spermatogonia
Fragilis	+	+	+	?
C-Kit	+	+	-	+
DAZL	-/+	+	+	+
Stella	-	+	+	+
Vasa	-	+	+	+
Miwi/Mili	+	+	+	+
Mil1	-/+	+	+	+
Mil2	-	+	+	+
TNAP	+	-	-	-
SSEA-1	+	-	-	-
Oct-3/4	+	+	+	-
α_6/β_1 integrin	-	+	+	-
Thy-1	?	+	+	-
Plzf	+	+	+	-
GCNA1	-	+	+	+
RET	?	?	+	-
GFR α 1	?	?	+	-
NCAM	?	+	+	?

2.2. Meiotic and post-meiotic phase of spermatogenic cells

Mature mammalian gametes must be haploid. To achieve this reduction in chromosomes, the germ cells undergo meiosis (Figure 1.4). With the onset of puberty, spermatogonia begin meiosis and give rise to the preleptotene spermatocytes. During prophase, the sizes and the nuclei of cells will progressively increase as the preleptotene spermatocytes progress into leptotene, zygotene, pachytene, and diplotene primary spermatocytes (Figure 1.5) (Russell and Frank 1978). These primary spermatocytes undergo meiotic DNA replication and recombination of homologous chromosomes. The diplotene spermatocytes undergo metaphase, anaphase, and telophase to complete the first meiotic division, or meiosis I. The cells now become secondary spermatocytes that will undergo the second meiotic division, or meiosis II, resulting in haploid spermatids (Russell, Ettlín *et al.* 1990). These spermatids are initially small round cells with a pale nucleus and a moderate amount of cytoplasm. However, during the third phase of spermatogenesis, or spermiogenesis, the round spermatids undergo extreme transformations into the complex form of spermatozoa including condensation of the nucleus, formation of the acrosome and flagellum, elimination of cytoplasm, and the arrangement of its mitochondria into a helix (Setchell 1978). This process is undergone without cell division and results in the reduction of the volume of the spermatid by approximately 25 % of its original size.

2.3. Microenvironment of testis

All adult stem cells can maintain a 'steady state' – generating one replacement stem cell and one differentiated progeny cell with no limit. Their specialized functions depend upon their neighboring differentiated cells by

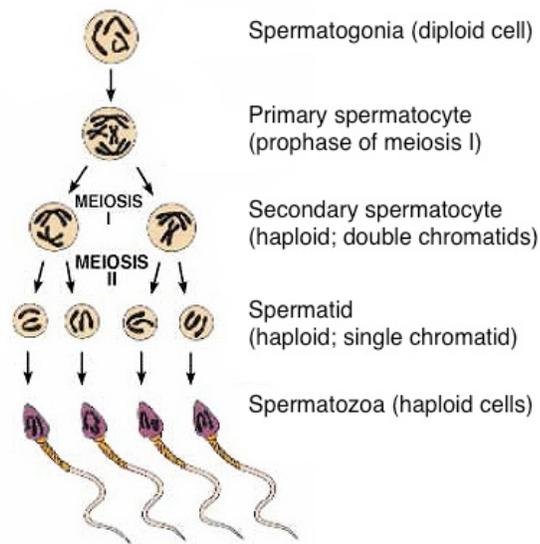


Figure 1.4. Meiosis of spermatogenic cells. Chromosomes are recombined and genetic material is halved in each sperm as a result of the two meiotic divisions.

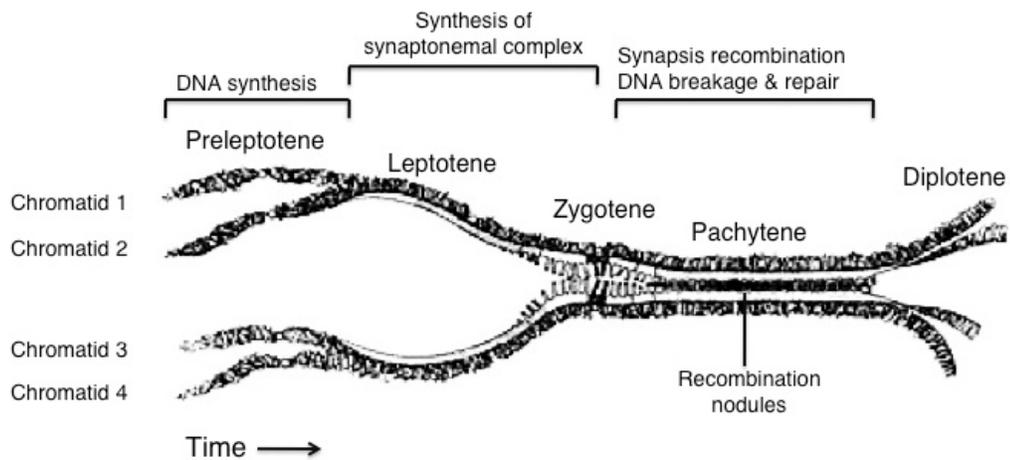


Figure 1.5. Schematic diagram of a pair of homologous chromosomes during prophase. In leptotene, the chromosomes begin to condense into long strands but remain unpaired. The homologous chromosomes become paired in synaptonemal complexes during zygotene. In pachytene stage, the chromosomes are fully lined up and genetic recombination takes place, by randomly exchanging segments of genetic information between nonsister chromatids (chromatids 1/2 vs. 3/4) at 'recombination nodules'. In diplotene, the synaptonemal complex dissipates, allowing the chromosomes to separate, except at chiasmata, where the crossing-over occurred.

signals and intercellular interactions. Consequently, location is as significant in the characterization of stem cells as are the special patterns of gene expression of the cells. The particular microenvironments that might control stem cells are called 'niches'. A niche can be defined as a subset of cells and extra-cellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production in vivo (Spradling, Drummond-Barbosa *et al.* 2001). Spermatogonial stem cells are located on the basal membrane of the seminiferous tubules, surrounded by Sertoli cells, the somatic supporting cells for this lineage (Figure 1.6). These cells produce various growth factors that regulate SSC self-renewal as well as differentiation. Glial cell line derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF-2) support self-renewal while activin A, bone morphogenic protein 4 (BMP 4) and stem cell factor stimulate differentiation of SSCs (Bugeaw, Sukhwani *et al.* 2005; Goriely, McVean *et al.* 2005; Kubota, Avarbock *et al.* 2004; Mauduit, Hamamah *et al.* 1999; Meng, Lindahl *et al.* 2000; Naughton, Jain *et al.* 2006). It has been shown that SSCs are not randomly distributed on the basement membrane of the tubule but are preferentially located in the area close to the interstitial tissue (Chiarini-Garcia, Hornick *et al.* 2001; Yoshida, Sukeno *et al.* 2007). A recent study by Shinohara *et al.* (Kanatsu-Shinohara, Takehashi *et al.* 2008) showed that β 1-integrin, an extracellular matrix component on SSC and Sertoli cells, plays an important role not just in migration of SSC into the niche but also in differentiation of SSC.

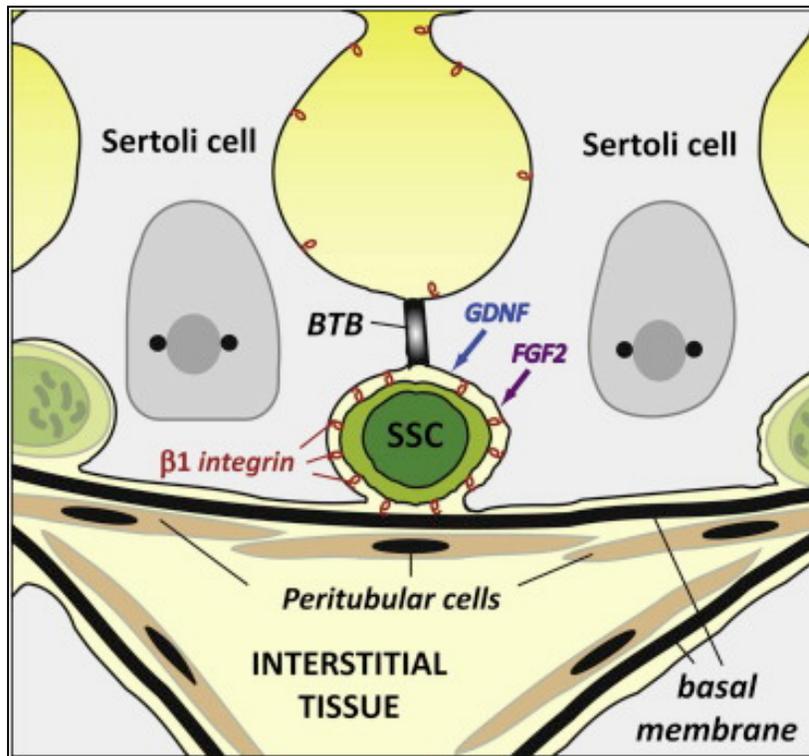


Figure 1.6. The spermatogonial stem cell niche. SSC (green) are located on the basal membrane of the seminiferous tubule in close contact with Sertoli cells (gray). Sertoli cells produce GDNF and FGF2 for regulating self-renewal of SSCs. To the lumen side of the SSC, the blood-testis barrier (BTB) is formed by tight junctions between neighboring Sertoli cells. The SSC niches can be found in those regions of the tubules that border interstitial tissue. β 1-integrin plays an important role in homing of the SSCs and may be involved in regulation of SSC differentiation. Reprinted from Cell Stem Cell, 3(5), de Rooij, 467-468, Copyright (2008) with permission from Elsevier.

Applications of male germline stem cells

1. Testis xenografting

Testis xenografting is typically performed by putting 1-2 mm³ sized pieces of testicular tissue into immunodeficient mice. Spermatogonial stem cells remaining in the testis tissue can survive in the recipient and eventually they produce sperm, supported by local testosterone produced under the regulation of the recipient mouse's endocrine system. This technique is a promising tool to study spermatogenesis as well as preserve male genetic information. Currently, sperm cryopreservation is the most common method to preserve male reproductive materials; however, it is not always possible to collect the male gamete, such as in the case of pre-pubertal males. Therefore, testis xenografting becomes very valuable for males that might die before ever producing sperm or who might need chemotherapy that would cause loss of their SSC. Even though testis xenografting is a straightforward technique, there are several considerations to perform it. First, prior to the current studies, it was unclear whether testis tissue from donors of different ages could support spermatogenesis as xenografts. Secondly, because only testis tissue is grafted, the sperm cannot undergo further maturation in the epididymis, which is essential for them to fertilize. Therefore, sperm produced by xenografts must be injected directly into oocytes, through the process of intracytoplasmic sperm injection (ICSI), to produce offspring. Although testis xenografting has been performed with various donor species including rodents (Schlatt, Honaramooz *et al.* 2003), farm animals (Rathi, Honaramooz *et al.* 2005; Rathi, Honaramooz *et al.* 2006; Zeng, Avelar *et al.* 2006), companion animals (Kim, Selvaraj *et al.* 2007; Snedaker, Honaramooz *et al.* 2004), primates (Honaramooz, Li *et al.* 2004; Rathi, Zeng *et al.* 2008) and humans (Geens, De

Block *et al.* 2006; Schlatt, Honaramooz *et al.* 2005), only a few studies have used sperm generated from xenografts to produce either offspring [mouse (Ohta and Wakayama 2005; Schlatt, Honaramooz *et al.* 2003)] or embryos [rhesus monkey (Honaramooz, Li *et al.* 2004) and pig (Honaramooz, Cui *et al.* 2008)]. There is no doubt that spermatogonial stem cells are maintained and supported to produce advanced germ cells in xenograft; however, further investigation is required to verify whether the sperm produced by the xenografts are normal in being able to fertilize oocytes and lead to normal embryo development. Furthermore, once xenografts are implanted in the recipients, there is only a limited timeframe to retrieve the sperm. To expand the potential of this technique, it should be studied whether frozen-thawed sperm collected from grafts could fertilize and contribute to normal embryo development. Alternatively, it may be possible to extend the time window by freezing testis tissue until it is needed (Jahnukainen, Ehmcke *et al.* 2007).

2. Spermatogonial stem cell transplantation

Spermatogonial stem cell transplantation (SSCT) was first reported in the mouse (Brinster and Zimmermann 1994), and it remains a powerful tool to verify the actual functional ability of putative SSC to produce sperm. In this technique, either populations of spermatogonia, or mixed germ cell populations containing spermatogonia, are placed within the lumens of the seminiferous tubules of a recipient. Placement is performed either by retrograde injection through the efferent ducts (rodents (Ogawa, Arechaga *et al.* 1997)), or via retrograde injection into the rete testis (large animal models (Honaramooz, Behboodi *et al.* 2003; Honaramooz, Megee *et al.* 2002)). Xenogeneic SSCT, in which the donor and recipient are different species, has

been performed using several species as donor and mice as recipients (Clouthier, Avarbock *et al.* 1996; Ogawa, Dobrinski *et al.* 1999). However, if the phylogenetic distance between the donor and recipient is too wide, the donor spermatogonia can colonize but spermatogenesis will not occur (Dobrinski, Avarbock *et al.* 1999; Kim, Selvaraj *et al.* 2006; Nagano, McCarrey *et al.* 2001). Therefore, different recipient species must be established for different donor species.

A recipient animal should have its endogenous germ cells depleted, so that the introduced cells will have improved access to the basal compartment of seminiferous tubules and so that there is a higher relative yield of donor-derived sperm (Brinster, Ryu *et al.* 2003). Two techniques have been widely used to reduce or deplete endogenous male germ cells as recipient preparation for SSCT: focal irradiation (Honaramooz, Behboodi *et al.* 2003; Izadyar, den Ouden *et al.* 2003; Schlatt, Foppiani *et al.* 2002) and chemotherapeutic drugs (Brinster, Ryu *et al.* 2003; Ogawa, Arechaga *et al.* 1997). SSCT has been used for various species such as pig (Honaramooz, Megee *et al.* 2002), monkey (Schlatt, Foppiani *et al.* 2002), goat (Honaramooz, Behboodi *et al.* 2003), cow (Herrid, Vignarajan *et al.* 2006) and dog (Kim, Turner *et al.* 2008). Transplantation of SSC from wild-type mice into the testes of mice having genetic infertility showed successful restoration of spermatogenesis in the recipient testes (Rilianawati, Speed *et al.* 2003). In addition, SSCT can be used not only to restore male fertility but also to generate transgenic animals. Various research groups showed that transduced SSC can generate sperm carrying a transgene after transplantation, and these can eventually produce transgenic mice (Nagano,

Brinster *et al.* 2001), rats (Ryu, Orwig *et al.* 2006), and transgenic goat embryos (Honaramooz, Megee *et al.* 2008).

3. Pluripotent stem cells derived from male germline stem cells

During normal development, SSC are restricted to differentiate into a single cell type, spermatozoa; however, several studies showed that SSC can give rise to embryonic stem cell-like cells in certain culture conditions, demonstrating the potential pluripotency of SSC. As reviewed in the previous section, Kanatsu-Shinohara *et al.* cultured SSC (Kanatsu-Shinohara, Ogonuki *et al.* 2003) in the presence of several growth factors including GDNF, which might be the most important factor in the regulation of self-renewal and proliferation of SSC (Braydich-Stolle, Nolan *et al.* 2005; Kubota, Avarbock *et al.* 2004; Meng, Lindahl *et al.* 2000). However, they also observed other kinds of colonies that were different from germline stem cells but similar to embryonic stem (ES) cell colonies. Further investigation showed that these colonies could be maintained in standard ES cell medium and had the same properties as ES cells, including expression of ES cell markers, and formation of teratomas, embryonic bodies and chimeras (Kanatsu-Shinohara, Inoue *et al.* 2004). Different groups have derived pluripotent stem cells from spermatogonial stem cells in modified culture conditions (Guan, Nayernia *et al.* 2006; Seandel, James *et al.* 2007). Interestingly, it does not seem to require a very specific approach to transform SSC into ES-like cells. This transition can occur on different feeder cells or even without feeder layers, substituted by leukemia inhibitory factor (LIF). In addition, the culture medium can also be varied while still supporting the transformation, as the groups of Kanatsu-Shinohara *et al.* (Kanatsu-Shinohara, Inoue *et al.* 2004) and Seandel *et al.*

(Seandel, James *et al.* 2007) used a stem cell medium whereas Guan *et al.* (Guan, Nayernia *et al.* 2006) used DMEM, although all three groups used GDNF. The ES-like cells derived from SSC were able to differentiate into specific cell lineages such as cardiomyocytes (Guan, Wagner *et al.* 2007) and endothelial cells (Baba, Heike *et al.* 2007). The earlier studies of the transformation were obtained in mice; however, recent studies showed that adult human germline stem cells could generate pluripotent stem cells as well (Conrad, Renninger *et al.* 2008; Kossack, Meneses *et al.* 2008). Therefore, the plasticity of male germline stem cells allows one to derive ES-like cells. The implications of this might well prove to be of importance for biomedical research and future clinical applications.

The domestic cat and dog as research models

Laboratory rodents have been widely and successfully used to understand mammalian biology. Generating transgenic mice is usually the ultimate step in functional characterization of mouse and human genomic loci. However, this approach is not without its limitations. Because there are significant differences in genetic and physiological profiles between humans and mice, development of appropriate mouse models for a large number of critical human diseases has been difficult. In addition, the mouse is severely limited as a behavioral model because of its limited repertoire of social interactions. Therefore, developing other mammalian models such as the dog and the cat, which have a more similar genome, behavior, and physiology to humans, is highly desirable for human medicine and veterinary research.

The recently completed sequencing of the dog genome (<http://www.genome.gov/17515860>) enables the dog to become a plausible

genetic model. Over 360 naturally-occurring canine genetic diseases have been shown to have counterparts in humans, including various forms of cancer (Lingaas, Comstock *et al.* 2003), blindness (Acland, Aguirre *et al.* 2005), and orthopedic defects (Athanasίου, Agarwal *et al.* 1995). Moreover, several diseases have been shown to involve the same gene in both species, such as muscular dystrophy and bleeding disorders (Ostrander and Wayne 2005). More than 400 different breeds have been generated by humans and many of these breeds originate from only a few founders and/or have undergone population bottlenecks. Therefore, many purebred dogs have unique genetic and phenotypic traits including susceptibility to certain genetic diseases. Availability of large numbers of different breeds allows researchers to use linkage analysis to identify genes responsible for traits and diseases. Once the genes involved in canine genetic diseases are identified using this approach, transgenic dog models would greatly facilitate our understanding of gene function and ultimately, the development of therapies and cures for the disease.

Like the dog, the cat has served as a research model for decades. Nearly 272 hereditary disorders have been found in domestic cats (Murphy 2006). Many of them result from the establishment of fifty domestic cat breeds in a short period of time. Almost a third of the diseases are unique to cats, having no counterpart in mice and dogs, which make them unique models for those diseases. The cat model has critically contributed in neurological research because a large number of characterized feline diseases are lysosomal storage diseases which have manifestations as human neurological disorders (Crawley, Brooks *et al.* 1996; Haskins, Casal *et al.* 2002; Haskins, Jezyk *et al.* 1980; He, Lowrie *et al.* 2005; Rabinowitz, Sheridan *et al.* 1991;

Wenger, Gasper *et al.* 1986; Yogalingam, Bielicki *et al.* 1997). In addition, the cat model has great potential for studies of infectious diseases and reproductive biology. Feline immunodeficiency virus (FIV) (Kanzaki and Looney 2004) and feline infectious peritonitis virus (FIPV) (Pears Wilkerson, Teeling *et al.* 2004) are the most notable diseases that have counterparts in humans--human immunodeficiency virus (HIV) and SARS coronavirus, respectively. Because many felid species have very poor sperm quality (Pukazhenti, Wildt *et al.* 2001), teratospermic felids can be models for human male infertility. The feline genome sequencing is at 2x coverage, which is far behind the dog genome sequencing project. However, feline geneticists have verified the causative mutations on coat color and disease phenotypes (Murphy 2006). As the genome sequencing progresses, linkage analyses and functional studies to identify the responsible genes for complex diseases will be accelerated, which will likely result in greater demand of the cat as a research model.

Research on both domestic dogs and cats can play significant roles in wildlife conservation efforts as well as being applied to human health. About 21 % of mammals are “threatened” or “endangered,” and the number of species so listed continues to grow larger every year (<http://www.iucn.org/redlist>). Since the physiology and pathology of domestic dogs and cats resemble greatly those of wild canids and felids, domestic carnivores can be excellent models for wildlife conservation applications.

Objectives

The aims of this dissertation are to optimize the stem cell-based reproductive technologies of SSCT and testis xenografting in the cat and dog, and to investigate the potential applications of these techniques, with a special emphasis on canine transgenesis. To facilitate the development of canine transgenesis, this dissertation includes investigations of female reproduction in the dog, because increased knowledge is needed in the “downstream” technologies of estrous synchronization, and embryo collection, manipulation, and transfer.

The specific objectives of my research are as follows:

- To optimize testis xenografting in the cat, first identifying the effect of the age of donor tissue, and then characterizing the functional abilities of sperm produced by xenografts
- To compare the success of testis xenografts using donor tissue from the ferret and dog
- To optimize spermatogonial stem cell transplantation in the cat and dog
- To investigate estrous synchronization in dogs, as well as embryo manipulation and transfer

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CHAPTER 2

Feline testicular xenografting:

Effect of donor age on success of spermatogenesis in feline testis xenografts*

and

Function of sperm produced in the xenografts †

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† Kim, Y., Commizoli, P., Pukazhenth, B. and Travis, A. J. Function of sperm produced in feline testicular xenografts. In preparation

Abstract

Ectopic xenografting of “donor” feline testicular tissue into a “recipient” immunodeficient mouse is a promising tool to preserve the male genome from genetically valuable felids. To define parameters under which the technique can succeed, we compared the effect of donor age on xenograft spermatogenesis among 4 age groups of domestic cats (*Felis catus*; 8 weeks to 15 months). In all cases, fresh tissue was grafted into castrated mice and collected 10, 30 and 50 weeks later. The percentage of xenografts recovered decreased as donor age increased. Mature testicular sperm were observed in xenografts from the 8 and 9-16 week age groups; only a single 7 month-old donor produced elongating spermatids, and xenografts from donors ≥ 8 months old degenerated. Seminal vesicle weight, an indicator of bioactive testosterone, was not significantly different among donors aged 8 weeks to 7 months versus controls, suggesting that xenograft Leydig cells were ultimately functional even in the 5–7 month age group. Regardless of donor age, production of mature sperm from xenografts was markedly delayed versus controls. Comparison of xenografts that produced sperm versus normal controls revealed a decrease in tubule cross-sections having post-meiotic germ cells. Based on the results of donor age effect, testis tissue from kittens younger than 4 mos old was used to study the properties of sperm produced in xenografts. We performed intracytoplasmic sperm injection (ICSI) to test their ability to contribute to normal embryo development. The cleavage rate of xenograft sperm-injected oocytes was 52.6% compared to 66.7% and 75% with fresh and frozen sperm-injected oocytes, respectively. None of the xenograft sperm-injected oocytes developed above 16 cell-stage, whereas

37.7% of control oocytes developed to 16 cell- or blastocyst stages. Together, these results indicate that the maximum practical donor age was just prior to onset of puberty and that even successful xenografts had abnormalities in spermatogenesis. Furthermore, xenograft produced sperm had poor fertility compared to control testicular sperm, necessitating further investigation of the function of xenograft sperm.

Introduction

The loss of genetic diversity due to infertility or the death of rare individuals is a critical obstacle to the conservation of endangered species, as well as to the maintenance of lines of research animals used to study inherited disease. The development of techniques to preserve genetic information from individual males has been the focus of much research (Pukazhenthil, Comizzoli *et al.* 2006). Historically, much emphasis has been placed on sperm cryopreservation; more recently, however, attention has also turned to other cell types within the testis.

During early testis development, gonocytes migrate toward the periphery of the seminiferous cords and transform into spermatogonia on the basement membrane (Bellve, Cavicchia *et al.* 1977). The term “spermatogonia” encompasses both undifferentiated cells, the spermatogonial stem cells (SSC), and a differentiating population that will enter meiosis and produce sperm (Ravindranath, Dettin *et al.* 2003). Because they are present from neonatal life into adulthood, SSC can be collected from immature males as well as adults. For conservation purposes, use of these cells therefore provides a potential complement to the cryopreservation of sperm, which can

only be recovered from mature individuals. In addition, as stem cells, the SSC have the ability to self renew, which terminally differentiated sperm cannot do. Recently, two new techniques, spermatogonial stem cell transplantation (SSCT) and testis xenografting, have been developed to take advantage of these attributes. In SSCT, “donor” male germ cells are transplanted into the testis of a “recipient” whose endogenous germ cells have been depleted or are absent (Brinster and Zimmermann 1994). It has been shown that the donor SSC can colonize and give rise to functional spermatozoa in a number of species (mice (Brinster and Avarbock 1994; Ogawa, Dobrinski *et al.* 2000), rats (Ryu, Orwig *et al.* 2003) and goats (Honaramooz, Behboodi *et al.* 2003)). In these successful cases, the donor and recipient have either been the same species or closely-related species, such as the rat and mouse (Franca, Ogawa *et al.* 1998; Shinohara, Kato *et al.* 2006). Therefore, this technique could be a promising tool for restoration of male fertility or for studies of spermatogenesis. However, if the taxonomic distance between donor and recipient is great (such as rabbit and dog into mouse (Dobrinski, Avarbock *et al.* 1999)), then xenotransplantation of stem cells is typically not successful. This limits the broad practical application of this technique until domestic animal models can be tested and optimized for suitability as recipients (Kim, Selvaraj *et al.* 2006).

Xenografting of testis tissue is another technique that relies upon the attributes of SSC to preserve male genetic information, but which preserves the architecture of the donor testis. This technique is performed by transplanting mm-sized pieces of testis tissue from a variety of species into immunodeficient mice, in which the xenografts can grow and produce sperm of the donor species. It has been performed with tissue from various donor species, including pigs and goats (Honaramooz, Snedaker *et al.* 2002),

hamster and monkey (Schlatt, Kim *et al.* 2002), cattle (Oatley, Reeves *et al.* 2005; Rathi, Honaramooz *et al.* 2005), rabbits (Shinohara, Inoue *et al.* 2002), cats (Snedaker, Honaramooz *et al.* 2004), humans (Schlatt, Honaramooz *et al.* 2005), and horses (Rathi, Honaramooz *et al.* 2006).

Interestingly, the nature of xenograft spermatogenesis varies tremendously between species, with xenografted tissue showing a decreased time to sperm production in the pig (Honaramooz, Snedaker *et al.* 2002) and primate (Honaramooz, Li *et al.* 2004), no difference in timing of sperm production in the bull (Oatley, de Avila *et al.* 2004; Rathi, Honaramooz *et al.* 2005), and remarkably delayed sperm production in the cat (Snedaker, Honaramooz *et al.* 2004). The factors that contribute to these species-specific responses are largely unknown, and could derive from the xenograft germ cells or somatic tissues, or from the hormonal interactions between the recipient mouse and the xenografts. In addition, differences in the age of the donor might also have effects on the ability of xenografts to support spermatogenesis. In most species, full spermatogenesis can be observed in xenografts from neonatal donors (Honaramooz, Snedaker *et al.* 2002; Oatley, Reeves *et al.* 2005; Schlatt, Honaramooz *et al.* 2003; Snedaker, Honaramooz *et al.* 2004), whereas limited attempts with fully adult testicular tissue have not supported spermatogenesis (Arregui, Rathi *et al.* 2008; Geens, De Block *et al.* 2006; Rathi, Honaramooz *et al.* 2006; Schlatt, Honaramooz *et al.* 2005). Beyond this broad division, only two studies have examined the effect of donor age on xenograft success. Of these, work done in cattle focused exclusively on early pre-pubertal donors (Schmidt, de Avila *et al.* 2006), and work done on stallions primarily investigated the endocrine regulation of xenograft function (Rathi, Honaramooz *et al.* 2006), with age considered only minimally.

For testis xenografting to be a useful tool for the conservation of genetically valuable felids, it is imperative that the range of donor ages for which the technique will be successful should be established. Therefore, we investigated the effect of donor age on the success of spermatogenesis in xenografts from domestic cat testis tissue and the function of sperm produced from xenografts in embryo development. Our results provide an effective age range for donors and describe further the nature of the delay in sperm production in felid testis xenografts (which sets the cat apart from other animal models studied to date). In addition, this study gives insight into how donor age affects testis xenograft success and whether sperm derived from xenografts can induce normal embryo development.

Materials and Methods

Experimental design

The first part of this chapter is to verify the effect of donor age in the cat testes xenografts. Because of the age range of donor tissue used in a previous study (Snedaker, Honaramooz *et al.* 2004), we concentrated our efforts on the use of donors greater than 5 weeks in age. In our preliminary experiments, testis xenografts from fully adult donors (older than 1.5 years old) uniformly degenerated (data not shown). We therefore narrowed our study to 4 age groups: 6 - 8 weeks old (weaning-early juvenile), 9 - 16 weeks old (pre-pubertal), 5 - 7 months old (pubertal) and 8 - 15 months old (young adult). In practice, all our donors for the youngest group were 8 weeks in age, refining our definition of that group. These age groupings clearly distinguish the pre-pubertal from pubertal, because it has been shown that the first sperm can be

found within testes between the 5 - 7 months (Tsutsui *et al.*, 2004). The second section of this chapter describes tests of the function of sperm that are produced in xenografts. Testes were collected from kittens younger than 4 mos old and sperm were harvested from the xenografts collect 39 – 45 weeks after xenografting for further analysis.

Reagents

All reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted. Isoflurane (Abbott Laboratories, North Chicago, IL) was used for anesthesia and buprenorphine (Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) was used for analgesia in the experimental mice. For histology, 100% and 70% ethanol (Pharmco, Brookfield, CT) and hematoxylin and eosin (Electron Microscopy Sciences, Fort Washington, CA) were used. Dulbecco modified Eagle medium (DMEM) and minimum essential medium (MEM) were purchased from Invitrogen (Carlsbad, CA).

Animals and xenografting procedure

Donor testes from domestic cats (*Felis catus*, 12 and 11 cats were used for the first and the second studies, respectively) were obtained from routine castrations at local veterinary hospitals and shelters. All samples were stored and transported in sterile saline at 4°C, and used within 24 h after collection. The testes were washed in cold PBS and visible blood was removed by blotting. One third of each testis was incubated overnight in Bouin's fixative for use as a histological control. The remaining portion of each

testis was processed for xenografting.

Briefly, the tunica albuginea and rete testis were removed and the testis parenchyma was cut into specimens measuring 1.5 - 2 mm³. These were kept in DMEM containing 100 µg/ml streptomycin sulfate and 100 IU/ml penicillin on ice until grafting. Four to eight week old ICR/SCID mice for the first study (Taconic, Germantown, NY) were used as recipients. However, recipient mice were replaced by Ncr/Nude strain (Taconic) due to longer life expectancy for the latter strain. Anesthesia was induced and maintained with 1.5 - 3.5% isoflurane. Castration was performed via a midline abdominal approach and an incision was then made on the dorsal midline and 6 -10 xenografts were placed under the skin (3-5 on each side, approximately 1 cm lateral of midline, and evenly spaced between the shoulder to the hips). A 5 mm length of 6-0 silk (Ethicon, Somerville, NY) was used to mark the site of graft placement to facilitate retrieval, and to loosely tether the grafts to prevent movement. The dorsal incision was closed with skin staples (Braintree Scientific, Inc., Braintree, MA). At the end of surgery, buprenorphine (1 mg/kg) was used for analgesia. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University. Information regarding the number of donors and recipients for the first study can be found in Table 2.1.

Table 2.1. Experimental design

Age group	No. of donors ^a	Donor age	No. of mice ^b	No. of grafts ^c	Total grafts ^d
8 weeks	3	8 weeks	2	16	49
		8 weeks	2	16	
		8 weeks	2	17	
9-16 weeks	3	10 weeks	2	16	50
		14 weeks	2	17	
		16 weeks	2	17	
5-7 months	3	5 months	1	8	36
		5 months	1	8	
		7 months	2	20	
8-15 months	3	8 months	2	16	51
		12 months	2	19	
		15 months	2	16	

a: Total number of donors for each age group

b: Number of recipient mice used per donor

c: Total number of xenografts per donor

d: Total number of xenografts placed for each age group

Analysis of xenografts

Based on the findings of Snedaker (Snedaker, Honaramooz *et al.* 2004), we evaluated the progress of spermatogenesis at 10, 30 and 50 weeks after xenograft placement. At these times, 2 or more xenografts from multiple donors were retrieved from recipient mice. The collected xenografts were measured in size and fixed in Bouin's solution overnight. The fixed xenografts were washed out of the Bouin's solution into 70% ethanol, and then dehydrated in ethanol prior to embedding in paraffin and sectioning at 4 μ m thickness. After mounting on slides, each section was deparaffinized with xylene and hydrated with 100% and 70% ethanol and water, prior to staining with hematoxylin and eosin. The sections were scanned under an Eclipse TE2000-U microscope (Nikon, Melville, NY) and images were captured using a Retiga 1300 color camera (QImaging corporation, Burnaby, BC, Canada) or a Spot RT camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Xenograft cross-sections were evaluated for the appearance and architecture of seminiferous tubules and Leydig cells, and for the presence of meiotic cells and progression of germ cell differentiation. Xenografts that showed collapsed seminiferous tubules and did not contain germ cells at identifiable stages were considered to be degenerated. Because xenografts were collected at different time points when different stages of germ cell development could be expected, xenograft "success" was defined variably at 10 weeks if seminiferous tubules contained any evidence of meiosis, at 30 weeks if pachytene primary spermatocytes were clearly visible, and at 50 weeks if elongating spermatids were visible. This changing definition of success emphasizes the production of later stages of spermatogenesis

because the primary goal of this work is to produce elongating spermatids and mature sperm to be used for technologies of assisted reproduction. At least 10 histological sections of each xenograft were examined for the most advanced germ cell type in the seminiferous tubule cross-sections, and the percentage of tubule cross-sections with each stage of germ cell types was calculated. The xenografts that produced sperm were compared against testes from age-matched controls to determine whether spermatogenesis in xenografts was similar to in vivo spermatogenic cell development.

When all xenografts were retrieved, the seminal vesicles were harvested from the recipient mice and weighed as an indicator of bioactive testosterone. Data from four retired breeder SCID mice were used to provide a control of normal seminal vesicle weight.

Gamete collection and preparation

Feline ovaries were collected from local veterinary clinics and transported in PBS at 4 °C within 6 hrs after ovario-hysterectomy. Grade I immature oocytes (with homogeneous dark cytoplasm, surrounded by several layers of compacted cumulus cells) were harvested from sliced ovaries and then cultured in in vitro maturation (IVM) medium containing MEM with 0.1 mM L-Glutamine, 1.0 mM pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 mg/ml bovine serum albumin (BSA), 1 µg/ml follicular stimulating hormone (1.64 IU/ml, NIDDK-ovine FSH-18; National Hormone and Pituitary Program, Rockville, MD), 1 µg LH/ml (1.06 IU/ml; NIDDK-oLH-25; National Hormone and Pituitary Program), 1 µg/ml estradiol for 30 hrs in 50-µl microdrops (10 oocytes/microdrop) under equilibrated mineral oil at 38.5 °C in 5% CO₂ in air.

Testicular xenografts were harvested at 39 – 45 weeks after implantation. All recovered grafts were measured and transported in DMEM supplemented with penicillin and streptomycin at the same dose above at 4 °C within 24 hrs to the laboratory performing ICSI. Upon arrival, the sperm were harvested by mincing the graft tissue in complete HEPES-Ham, which was composed of HEPES-Ham F10 medium (Irvine Scientific, Santa Ana, CA) supplemented with 1.0 mM pyruvate, 2.0 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5% of FBS at room temperature. The sperm suspension was collected from the minced tissue and centrifuged at 300 x g for 8 min. The sperm pellet was resuspended in 50 µl of 0% glycerol-TEST egg yolk solution (Irvine Scientific) in a cryovial and then an equal volume of 8% glycerol-TEST was added to make a final concentration of glycerol at 4%. The mixture was cooled in a water bath for 2 h and then frozen 1 inch over LN2 vapor for 10 min. The frozen sperm were kept in LN2 tank until ICSI procedure. Adult cat testes were obtained from local veterinary hospitals to collect testicular sperm, which served as controls.

ICSI procedure

Frozen sperm were thawed in a water bath at 37°C and centrifuged at 300 x g for 8 min. The pellet was re-suspended for ICSI procedure in the complete HEPES-Ham. Five microliters of thawed xenograft sperm and either fresh or frozen control testicular sperm was mixed with an equal volume of 10% polyvinylpyrrolidone (PVP; Irvine scientific). The two different sperm drops were placed separately in the center of a 50 x 9 mm Petri dish and surrounded with four 10-µL drops of complete HEPES-Ham's containing in vitro matured oocytes (previously denuded by gentle pipetting in 0.2%

hyaluronidase). The dish was flooded with mineral oil and maintained on the heated stage (38.5 °C) of an inverted microscope (Olympus BX41, Olympus Corporation, Melville, NY) equipped with micromanipulators (Narishige, Sterling, VA), holding and micro-injection pipettes (Humagen Fertility Diagnostics, Inc., Charlottesville, VA). A single morphologically normal spermatozoon was selected from a sperm drop, aspirated and injected into the middle of an oocyte with a visible first polar body. After ICSI, oocytes were activated by incubation in complete Hepes-Ham's (containing 7% ethanol) for 5 min at 38.5 °C. After extensive rinsing, injected oocytes were cultured in vitro in complete Ham's without Hepes (38.5 °C, 5% CO₂ in air). At 7 days of culture, embryos were fixed and stained with Hoechst 33342 to assess blastomere numbers.

Statistical analysis

All data were analyzed using Origin 7.0 Software (OriginLab Corporation, Northampton, MA). ANOVA was performed to evaluate potential correlations between xenograft success and donor age and the difference in xenograft size at different times of collection. In addition, a Student's *t*-test was performed to compare the difference in spermatogenic cell development between the xenografts and normal cat testes. Statistical significance was considered at $p < 0.05$.

Results

Histological analysis

Table 2.2 shows that the percentage of xenografts recovered from recipient mice decreased as donor age increased, and the percentage of the xenografts which contained germ cells at different stages of spermatogenesis varied with both donor age and the retrieval time points. For the 8 week old and 9 - 16 week old donor age groups in our study, we had a percent recovery rate very similar to those reported for other species (Rathi, Honaramooz *et al.* 2005; Zeng, Avelar *et al.* 2006).

At the 10 week time point, spermatocytes were seen as the most advanced germ cell stage in successful xenografts from the 3 youngest donor groups: 8 week, 9 –16 week, and 5 – 7 months old (Figure 2.1B, 2.1F, and 2.1J, respectively). Seminiferous tubules in xenografts collected at this time from 8 – 15 month old donors had degenerated (Figure 2.1N). Early elongating spermatids were the most advanced germ cells in the xenografts of 9 – 16 weeks old donors (Figure 2.1G) at 30 weeks after xenografting, whereas spermatocytes were the most advanced germ cells in the xenografts from the 8 week old (Figure 2.1C) and 5 – 7 month old groups (Figure 2.1K) at this time point. In the 8 – 15 month old donor group, most of the xenografts retrieved at the 30-week time point had degenerated; however, one xenograft was found with rare spermatocytes that did not appear to be normal (Figure 2.1O). Complete spermatogenesis, defined by the presence of mature testicular sperm, was observed in 13.3 % and 16.6 % of the recovered xenografts from the 8 week old (Figure 2.1D) and 9 – 16 week old groups (Figure 2.1H), respectively, at 50 weeks post procedure. Elongating spermatids were found

as the most advanced stage of germ cell development from one 7 month-old donor (Figure 2.1L), whereas xenografts from donors 8 months of age or older were invariably degenerated at 50 weeks post implantation (Figure 2.1P).

Table 2.2. Effect of donor age on parameters of spermatogenesis in feline testis xenografts

Age group	Grafts Recovered (%) [^]	Time after grafting (weeks)	Most advanced germ cell stages in xenografts (%) [*]				
			SG	SC	RS	ES	Sperm
8 weeks old	81.6	10	87.5	37.5	-	-	-
		30	73.3	33.3	-	-	-
		50	80.0	80.0	40.0	20.0	13.3
9-16 weeks old	72.0	10	80.0	80.0	-	-	-
		30	100	100	57.1	28.6	-
		50	100	100	70.8	50.0	16.6
5-7 months old	63.8	10	50.0	25.0	-	-	-
		30	42.8	42.8	-	-	-
		50	87.5	50.0	25.0	12.5	-
8-15 months old	33.0	10	-	-	-	-	-
		30	9.0	9.0 [#]	-	-	-
		50	-	-	-	-	-

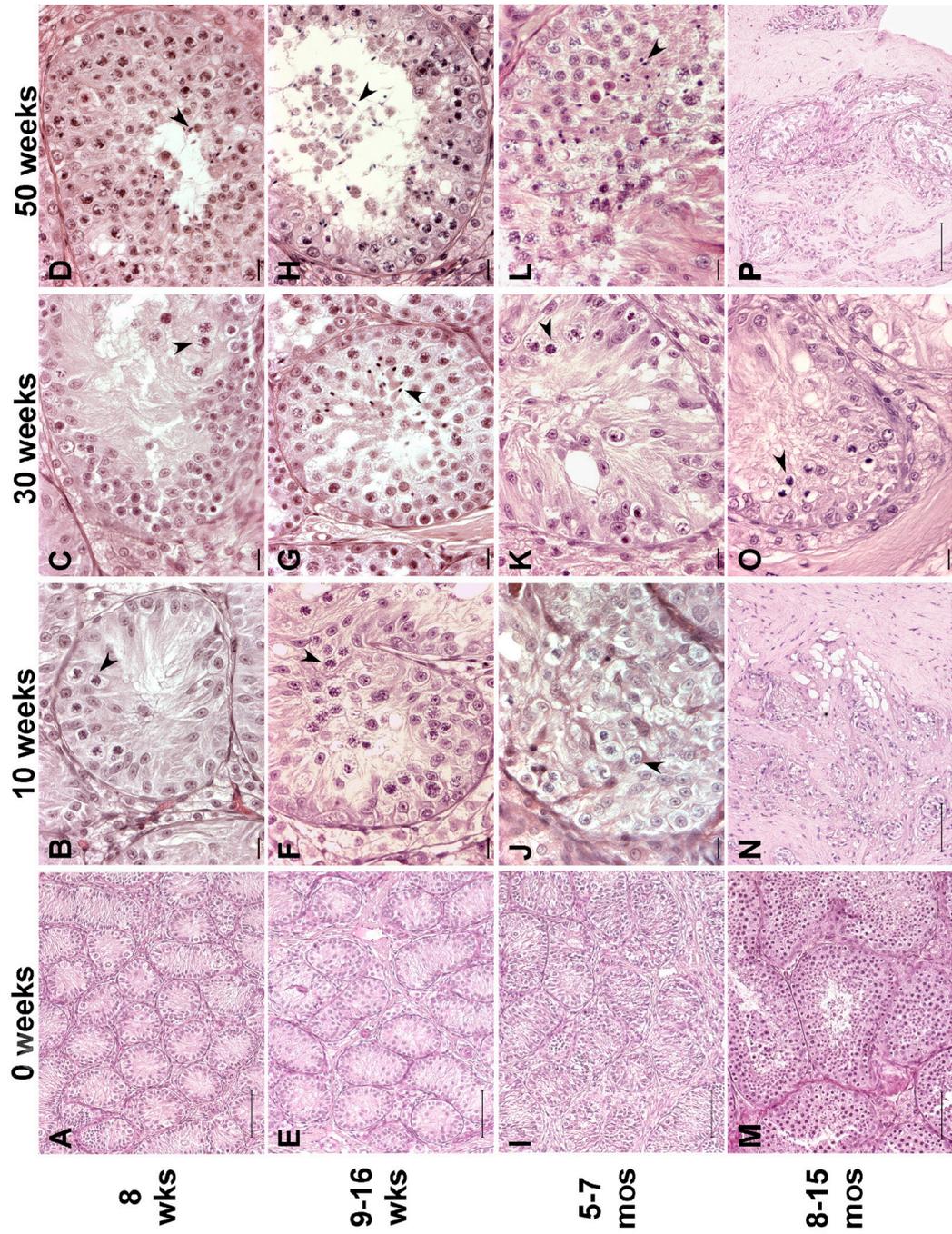
[^]: Total number of xenografts retrieved divided by the total number of grafts placed

^{*}: Total number of xenografts which contained the indicated stage of germ cell development divided by the total number of xenografts retrieved at that time point, SG: spermatogonia, SC: spermatocytes, RS: round spermatids, ES: elongating spermatids, Sperm: spermatozoa

[#]: The spermatocytes in this section did not appear to be normal.

Figure 2.1. Cross-sections of xenograft seminiferous tubules.

Panels shown under “0 weeks” are representative of the histological appearance of donor tissue at the time of xenografting. Panel A represents an 8 week old donor; panel E a 14 week old donor; panel I a 5 month old donor; and panel M a 12 month old donor. Xenografts were collected from the different donor age groups [8 weeks (B, C, D), 9 - 16 weeks (F, G, H), 5 - 7 months (J, K, L) and 8 - 15 months (N, O, P)] at 10, 30, and 50 weeks post-procedure. Spermatocytes (arrow heads in these panels) were the most advanced cells from the xenografts collected at 10 (B, F, J) and 30 weeks (C, K, O) post grafting. Elongating spermatids (arrow heads in these panels) were the most advanced germ cells from the 9 - 16 week donor xenografts collected at 30 weeks (G), and from the 5 - 7 month donor xenografts collected at 50 weeks (L) post procedure. Arrowheads in panels D and H show spermatozoa as the most advanced germ cells in the xenografts collected at 50 weeks for the 8 week and 9 - 16 week donor age groups. Xenografts from young adult donors were all degenerated at 10 weeks (N) and 50 weeks (P) after xenografting, although apparently abnormal spermatocytes were seen rarely from one donor at the 30-week time point. Bar = 15 μm except for panels A, E, I, M, N and P (100 μm).



Evaluation of xenograft testosterone production

Seminal vesicle development is highly androgen dependent (Setchell 1978). Therefore, in the castrated recipients, it is commonly used as a marker for xenograft production of bioactive testosterone (Rathi, Honaramooz *et al.* 2006; Schlatt, Honaramooz *et al.* 2003; Schmidt, de Avila *et al.* 2006). The average weight of recipient seminal vesicles varied only slightly among the 3 youngest donor age groups (174 ± 41.2 mg for 8 week old donors; 157 ± 53.7 mg for 9 - 16 week old donors; and 215 ± 29.2 mg for 5 - 7 month old donors, Figure 2.2), whereas a significant decrease was seen in seminal vesicle weight of recipients of the 8 - 15 month old donor tissue (23.5 ± 9.6 mg). When compared against the seminal vesicle weights of control SCID mice (294.84 ± 16.3 mg), only the latter group showed a statistically significant difference ($p = 0.001$). Of note, seminal vesicle weights in the recipients of 5 - 7 month old

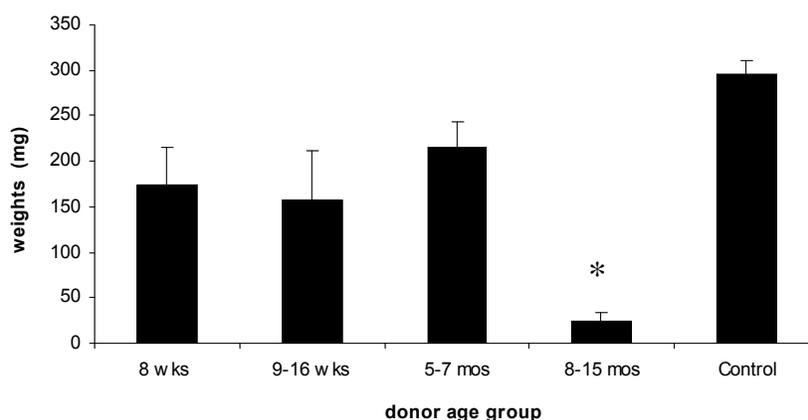


Figure 2.2. Seminal vesicle weight for each donor age group. Seminal vesicles were collected and weighed at 50 weeks post xenografting and were compared against control tissue from intact retired breeder SCID mice ($n = 4$). An asterisk (*) indicates a significant difference ($p < 0.05$) between the 8 - 15 month old age group and the control. Data are presented as mean \pm SEM.

donor tissue were similar to those in the younger donor age groups even though the xenografts in the 5 - 7 month old donor group were rarely successful at the 50 week time point.

Effect of donor age on spermatogenesis in testis xenografts

Per collection time point, we pooled the size data from what we defined as successful versus unsuccessful xenografts and found a significant difference between the two for the 10- and 50-week time points ($p = 0.006$ and 0.04 , respectively), with successful xenografts being larger than unsuccessful xenografts. When data from all three time points were pooled, the size difference between successful and unsuccessful xenografts was also significant ($p < 0.0001$). However, when looking at the data as a scatter plot (Figure 2.3A and 2.3B), with individual xenografts distinguished for age group and outcome, one can see that larger xenograft size at the time of retrieval was not entirely predictive of success, nor was small xenograft size entirely predictive of failure (Figure 2.3B). Expansion of Leydig cells and seminiferous tubules that contained only Sertoli cells contributed to the size of large, but unsuccessful, xenografts (data not shown).

Even in successful xenografts, there appeared consistently a subset of seminiferous tubules that contained no germ cells (Figure 2.4). At the 50-week time point in xenografts showing complete spermatogenesis, $20.4 \pm 1.6\%$ of seminiferous tubule cross-sections contained no germ cells (Figure 2.4A and 2.5A). Data in Figure 2.5 were calculated in two ways (Groups A and B), and those data in group B were compared with a control group (C). Percentages in group A were based on the total number of seminiferous tubule cross-

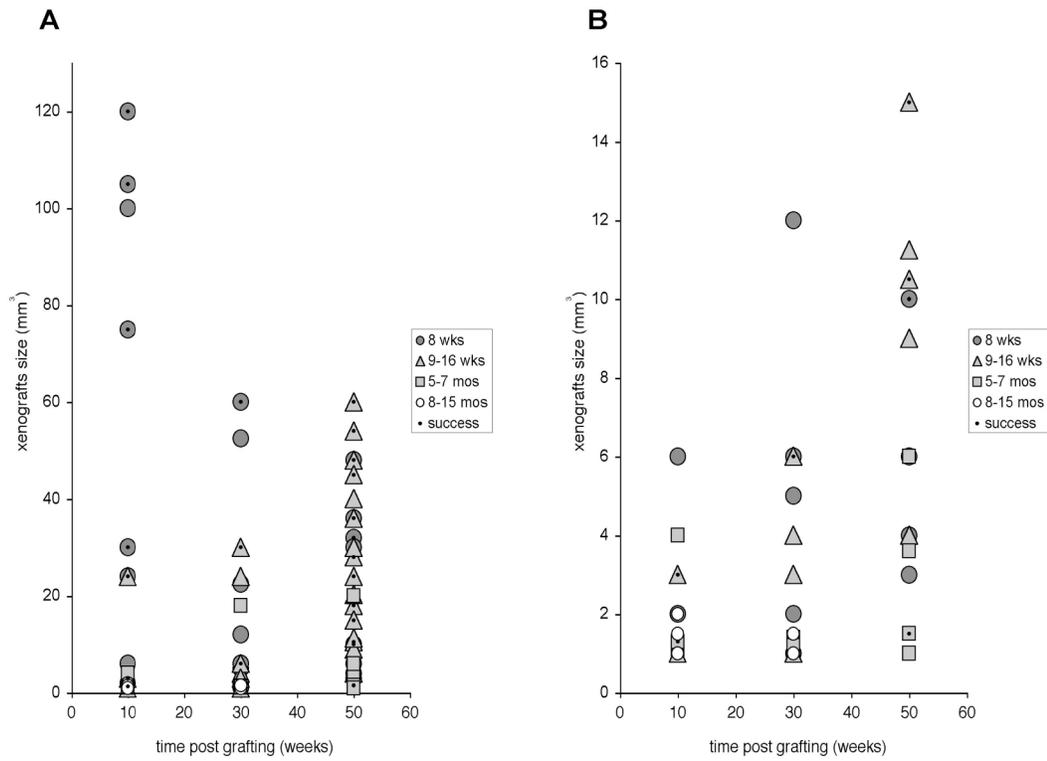


Figure 2.3. Scatter plot of xenograft size and outcome. Xenografts were collected from each donor group at three different time points post procedure and measured. Panel B is a close-up view of smaller sized xenografts (0 – 16 mm³) from panel A, to show that small size did not necessarily preclude success.

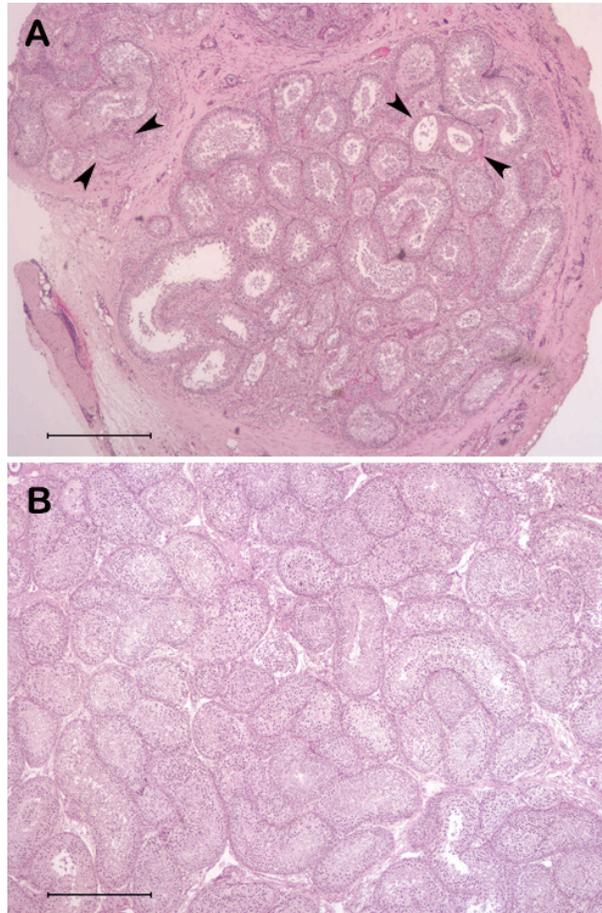


Figure 2.4. Cross-sections of a successful testis xenograft and a control testis. Panel A shows a xenograft at 50 weeks post-procedure from the 8 week-old donor group. Note the appearance of degenerating tubules (arrowheads) interspersed between tubule cross-sections containing meiotic germ cells. Note also the connective tissue capsule, which formed around the xenografts. Panel B shows a 12-month-old control. In both panels, the bar represents 500 μm.

sections, including those containing no germ cells. To compare spermatogenesis in the xenografts versus that in normal cat testes more accurately, we also determined percentages after having factored out the empty tubules (Group B). Even with this correction, a significant difference ($p < 0.001$) was observed in the pattern of spermatogenesis between tubules in xenografts versus normal testis from adult cats ($n = 3$). The percentages of tubules having spermatocytes and elongating spermatids were not significantly different, but the percentages containing round spermatids and sperm were significantly lower than those of controls ($p < 0.001$ for both). The percentage of tubules containing spermatogonia was also significantly different ($p < 0.001$), although the difference between the values was exceedingly small.

Analysis of xenograft sperm and embryo produced with the sperm

A total of 120 grafts were recovered out of 168 xenografts placed, at time points between 39 and 45 weeks after implantation. Sperm were found in 22 grafts (18%) and most of them showed morphological abnormalities, mainly microcephaly and missing midpieces. Nineteen morphologically normal xenograft sperm were retrieved from cryopreservation and injected into in vitro matured oocytes. As controls, 15 fresh and 12 frozen testicular sperm were also injected into oocytes. 52.6% of oocytes with xenograft sperm were cleaved which is not significantly different from the rates of fresh and frozen control sperm (66.7% and 75%, respectively). As shown in Figure 2.6, all xenograft sperm-injected oocytes arrested at earlier stages than morula, whereas frozen testicular sperm-injected oocytes developed up to blastocyst stage (7 morulae and 2 blastocysts) at 7 days post ICSI.

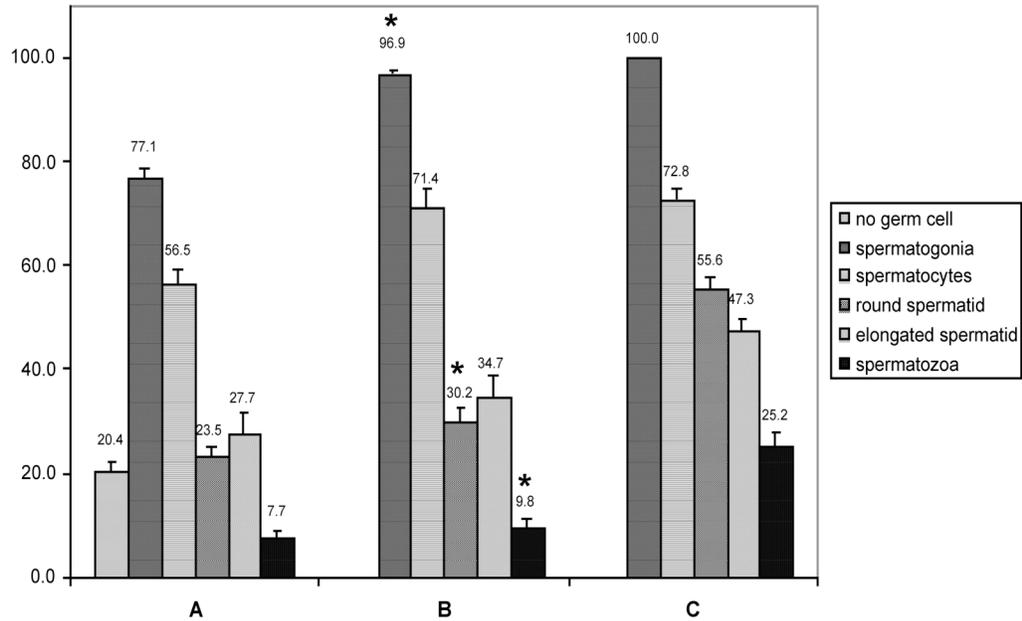


Figure 2.5. Percentage of seminiferous tubule cross-sections containing different stages of spermatogenic cells. Percentages in groups A and B were derived from three xenografts that produced morphologically mature sperm at the 50 week time point, and percentages in group C were derived from testes of three normal cats (9 - 15 months old). The distribution of spermatogenic cells in seminiferous tubules was calculated differently for the groups. In group A, the number of tubules containing each germ cell type (or no germ cells) was divided by the total number of tubule cross-sections ($n = 1777$) in the xenografts. Group B is similar to A, but the number of tubule cross-sections containing no germ cells was excluded from the total ($n = 1184$). In control group C, the number of tubule cross-sections containing each germ cell type was divided by the total number of cross-sections assessed ($n = 900$). Asterisks (*) indicate a significant difference ($p < 0.05$) between Group B and Group C. Data are shown as mean \pm SEM.

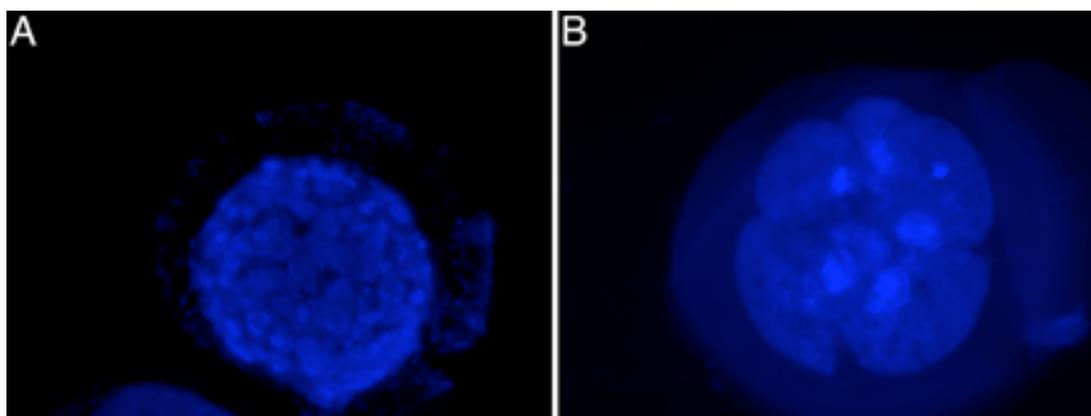


Figure 2.6. The embryos generated by ICSI. Panel A shows an early blastocyst (200 μm), generated with control testicular sperm, at day 7 in culture and panel B shows a xenograft sperm-injected embryo (150 μm) arrested at 8 cell-stage at the same day. Blue fluorescence labeled indicates DNA in the nuclei.

Discussion

Across species used in various xenografting studies, the variable of donor age has not been intensively studied, although defining the parameters under which the technique can be used successfully is a critical requirement for moving the technology to practical application, and for providing basic scientific understanding of the determinants of xenograft success and failure. A major finding of our study was that once meiotic cells appeared consistently throughout a donor testis, the ability of that tissue as a xenograft to support spermatogenesis declined dramatically. This was seen in two ways: for donors ≥ 8 months in age, there was complete degeneration of xenografts. For donors aged 5 - 7 months, the period when the first sperm are produced and spermatogenesis begins in the majority of tubules [(Tsutsui, Kuwabara *et al.* 2004), and data not shown], xenografts typically failed to support full spermatogenesis, although they did often support early meiosis up to spermatocytes. The findings of degenerating spermatocytes from one donor in the 8 - 15 month group, and of elongating spermatids from one donor in the 5 - 7 month age group, point out the presence of minor individual variations between samples. This perhaps represents focal variation in the timing of onset of spermatogenesis within a testis. For example, we have noted previously the presence of sperm in epididymides from 5 month-old cats, although no histological evidence for complete spermatogenesis was seen in the vast majority of testis cross-sections examined from those animals (Kim, Selvaraj *et al.* 2006). Conversely, the one 7-month old donor might have had a focal area that lagged behind the spermatogenic development seen in the rest of its testis, and the rest of the donors in its age group.

Interestingly, xenografts from the youngest donor group had an initial slight acceleration of the onset of meiosis compared to age-matched controls (compare Figure 2.1B and 2.1I). Spermatocytes were observed in approximately one-third of the xenografts 10 weeks post-procedure in the 8 week-old donor group, as opposed to typically appearing at age 5 - 6 months. This has been noted by others (Oatley, Reeves *et al.* 2005; Rathi, Honaramooz *et al.* 2005; Zeng, Avelar *et al.* 2006) working not only with species in which sperm production is accelerated, but also species in which sperm production occurs at approximately the same time as in age-matched control testes. The most likely explanation for this early onset of meiosis is that the xenograft tissue is suddenly exposed to an adult endocrine profile in the recipient mouse, and the germ cells are able to respond (Honaramooz, Snedaker *et al.* 2002; Rathi, Honaramooz *et al.* 2005; Schlatt, Honaramooz *et al.* 2003).

Despite this initial hastening, fully mature sperm were still not recovered until after they would have appeared in a normal testis. These findings potentially help narrow the underlying cause(s) of species differences in time until xenograft sperm production: regardless of donor species, it seems that xenografts are stimulated equally and initially can respond similarly when placed into an adult endocrine environment. However, the feline xenografts differ in that they do not become competent to support full spermatogenesis for some time. This suggests an immaturity of some component within the feline testis xenograft. Alternatively, the feline xenografts might have been completely mature with regard to their ability to maintain response, but a mismatch between donor and recipient regarding the endocrine environment resulted in effective hormonal support that was

borderline insufficient to maintain spermatogenesis. Only after a prolonged period was a relatively small subset of meiotic cells able to complete their differentiation.

In the 9 - 16 week donor group, spermatogenesis was initially slightly delayed versus age-matched control testis, but xenografts from this group had a more normal rate of spermatogenesis, with elongating spermatids seen by 30 weeks. It is therefore likely that had our study design included additional time points, we might have found mature spermatozoa in some xenografts earlier than 50 weeks. However, our experimental design was derived to maximize the chance of finding success, as opposed to finding the earliest time of xenograft success.

Differences in xenograft success between pre-pubertal and pubertal donor testes offer the possibility that inherent differences in the stem cell cohorts between these ages might contribute to success or failure in ability to support spermatogenesis. However, it is also possible that somatic cell components or later stages of germ cell development also factor prominently in the effect of donor age. Rathi (Rathi, Honaramooz *et al.* 2005) suggested that meiotic/post-meiotic cells might be less likely to survive the hypoxia associated with the procedure. This is logical given the requirements in rodents for oxidative metabolism as opposed to glycolysis in post-meiotic germ cells (Bajpai, Gupta *et al.* 1998; Grootegoed, Jansen *et al.* 1986; Nakamura, Okinaga *et al.* 1986), and could account for the loss of differentiating male germ cells seen soon after placement of donor tissue containing meiotic cells. Yet, once having been lost, the underlying complement of SSC should theoretically still have been the same as in pre-pubertal donors, and therefore

should still have been able to recover and repopulate to support spermatogenesis over the 50 week period. The failure of the xenografts to do so suggests that there were indeed differences in either the stem cell or somatic cell components of the testis that arose during puberty, and which affected the ability to recover from this initial loss. One potential endocrine-based rationale might be that once having been “entrained” to a normal feline endocrine profile in puberty, the pubertal or young adult feline testis was less adaptable to the murine endocrine profile than feline testes that had not yet been exposed to an increase in gonadotropins.

Unlike what was seen in the 5 - 7 month age group, where the xenografts often survived despite being unable to support spermatogenesis, xenograft failure in the young adult (8 - 15 month) group was more typically a complete failure in which the xenografts did not survive and were not able to be retrieved. This type of failure could represent alterations in growth/angiogenic factors in testicular somatic cells that led to global hypoxia and tissue damage. In addition, complete failure of Leydig cells or of the seminiferous epithelium, or some combination thereof, could have occurred.

Of practical importance, larger xenografts producing more sperm would have benefits in terms of ease of sperm recovery. Yet for use in procedures such as intra-cytoplasmic sperm injection, even low numbers of sperm could have significant impacts on genetic diversity within a population. In this regard, xenograft size was correlated with, but was certainly not entirely predictive of, either success or failure. Even in successful xenografts, many tubule cross-sections contained no evidence of germ cells. The finding that these cross-sections were not restricted to focal areas but could be found in

multiple places within a successful xenograft argues against regional microenvironmental factors, such as hypoxia or nutrient starvation on one side of a graft as contributing to the failure. Rather, success within a specific region of a tubule within a xenograft might depend on the timing of the spermatogenic wave within that region. To compare xenograft spermatogenesis more accurately versus normal controls, we factored out those cross-sections that did not support male germ cell development. Still, the remaining tubule cross-sections showed differences versus normal testis controls. In particular, the percentage of cross-sections containing stages of post-meiotic germ cells was decreased. This finding suggests meiotic arrest within many of the cross-sections, and these arrested tubules could then degenerate, leading to the empty tubules just discussed. Whether this suggestion of meiotic arrest points toward other changes in meiosis that were not detectable histologically is an extremely important question.

Although testing the quality and function of sperm produced by testis xenografts is an essential step, there have been a few reports about generating embryos or offspring using xenograft sperm. Honaramooz *et al.* (Honaramooz, Snedaker *et al.* 2002) demonstrated that sperm produced from mouse, pig and goat testicular xenografts can activate mouse oocytes and the study of Schlatt *et al.* (Schlatt, Honaramooz *et al.* 2003) showed that mouse progeny were produced with xenograft-derived sperm via ICSI. In the current study, we attempted ICSI with sperm produced from cat testicular xenografts to test their quality in supporting embryo development and eventually producing kittens. Because about 70% of felid species show teratospermia (ejaculation of <40% of morphologically normal sperm), and high numbers of domestic cats also are teratospermic (Pukazhenti, Wildt *et al.* 2001), we were

concerned about possible lack of reproductive fitness of donor animals, which would be undetectable at the early age required for successful xenografting. This concern proved to be justified, with only 27% of the donors producing sperm in the xenografts. Interestingly, using tissue from single donors, multiple recipients maintained successful xenografts that produced sperm, providing evidence that successful grafts depended more on the fitness of donor tissue rather than differences between individual recipients.

For our first trial of ICSI, sperm were collected at 48 weeks after xenografting. 54.5% of oocytes injected with these sperm cleaved but most of them degenerated at the 4-cell stage. In a xenograft, once the sperm mature, they will eventually degenerate because of the absence of an outflow tract (i.e. there is no epididymis). Therefore, the right timing to collect sperm must be optimized, which is not too long after their maturation but long enough so that the xenograft can produce enough sperm to be retrieved. Because our earliest observation of sperm in cat xenograft was around 39 weeks after implantation, we next collected xenografts at the different time points of 40 and 44 weeks to minimize degeneration of the sperm. None of the grafts collected at the 40 week time point produced sperm and the most of germ cells observed were spermatocytes (data not shown). A few sperm were found from the xenografts collected at 44 weeks. The majority of sperm were microcephalic or had missing midpieces and none of the embryos injected with these sperm could develop to blastocyst stage. However, even normal cat testicular sperm showed a lower success rate of morula-blastocyst development compared to ejaculated sperm (Comizzoli, Wildt *et al.* 2006a), which is not true with other species (Van Steirteghem, Nagy *et al.* 1998; Yanagimachi 2005). Comizzoli *et al.* (Comizzoli, Wildt *et al.* 2006b) improved ICSI success with cat testicular

sperm by co-injecting mature centrosomes collected from ejaculated sperm, which seems to be responsible for the first cell division of the embryo. As a next step in exploring the function of xenograft-derived sperm, our ICSI procedure will include this co-injection of a mature centrosome.

Neonatal or juvenile mortality is a significant problem in the captive management of threatened/endangered felids. This is particularly true for those species with limited founder populations, in which the breeding success of a single individual can have long-term implications for the genetic viability of that population. Our findings suggest that the testis xenografting technique represents a potentially important complement to current conservation efforts by providing a method to preserve the breeding potential of neonatal to pre-pubertal males. However, for this to be realized, additional work is needed to ensure that sperm produced are functional.

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CHAPTER 3

Comparative study of testis xenografting in the dog and the ferret*

Abstract

Testis xenografting is a promising tool to study spermatogenesis and an alternative means to preserve the genetic information in genetically valuable male animals. The present study was conducted to test the outcomes of this technique using testis tissue from different species of carnivore. In particular, we compared the progression of germ cell development in xenografts from neonatal- to pubertal-aged dogs and 8 week-old ferrets. Testis tissue was ectopically implanted under the skin on the backs of castrated nude mice and subsequently evaluated for testosterone production and establishment of spermatogenesis at various time points: 10, 30, and 50 weeks and 10, 20, 25, and 30 weeks after grafting, for the dog and ferret, respectively. 75% of dog xenografts were recovered whereas 40% of ferret xenografts were harvested. Seminal vesicles were collected from the recipient mice and weighed as an assay for bioactive testosterone. The weights of seminal vesicles from the mice of both species showed no significant difference from the value of uncastrated, control nude mice, indicating that the xenografts were producing testosterone. Germ cell development in the dog xenografts was quite poor, with almost no production of mature sperm regardless of the age of donor tissue. Conversely, the ferret testicular xenografts produced differentiated germ cells and sperm at the same time as the age-matched controls. These data demonstrate dramatic differences in the ability of testicular tissue from different donor species of carnivore to establish spermatogenesis following ectopic testicular xenografting.

* Please note that a portion of ferret xenograft procedures were performed by Dr. Jim Gourdon, and a detailed analysis of the ferret xenografts is to be

performed by him as his contributions to a co-authored manuscript that will incorporate the experiments and results described in this chapter.

Introduction

Spermatogenesis is a highly complex process to generate male gametes continuously throughout the lifetime of a male animal. This process begins with spermatogonial stem cells (SSC), which will undergo a dynamic process of proliferation and differentiation with extensive support from the somatic Leydig and Sertoli cells (Setchell 1978). The complexity of the system has been an obstacle to recapitulate and study spermatogenesis in vitro. In 1994, Brinster et al. introduced spermatogonial stem cell transplantation (SSCT) as the first functional assay of SSCs (Brinster and Zimmermann 1994), and this has greatly accelerated SSC research. Several years later, the technique of testis xenografting was reported as another promising tool to study spermatogenesis (Honaramooz, Snedaker *et al.* 2002).

In addition to facilitating research on spermatogenesis, these technologies can be used practically as tools to preserve male genetic information not only for wildlife conservation but also for human cancer patients. When a genetically valuable animal dies or a cancer patient needs harsh chemotherapy that can destroy his fertility, cryopreservation of sperm is the most common method used to conserve their genetic information for future use. Because sperm are terminally differentiated cells incapable of self-replication and are only produced from sexually mature animals, cryopreservation of sperm has some limitations. However, techniques using SSC can overcome these limitations: SSC can be harvested throughout an animal's lifetime after birth and they can produce sperm indefinitely. Therefore,

testis xenografting, which preserves spermatogonial stem cells, could provide a remarkable potential to preserve male genetic information.

Testis xenografting is performed by implanting 1-2 mm³ pieces of testis tissue into immunodeficient mice. Over time, the xenografts grow and produce sperm in the recipient. It has been shown that sperm produced from xenografts can produce offspring as well (Schlatt, Honaramooz *et al.* 2003). Since the first testis xenografting was performed in the mouse, various donor species have been used including pigs and goats (Honaramooz, Snedaker *et al.* 2002), hamster and monkey (Schlatt, Kim *et al.* 2002), cattle (Oatley, Reeves *et al.* 2005; Rathi, Honaramooz *et al.* 2005), rabbits (Shinohara, Inoue *et al.* 2002), cats (Snedaker, Honaramooz *et al.* 2004), humans (Schlatt, Honaramooz *et al.* 2006), and horses (Rathi, Honaramooz *et al.* 2006). In all cases thus far, a dramatic decline in success of xenografts has been noted when using adult tissue (versus pre-pubertal). Therefore most studies have utilized neonatal or pre-pubertal donor tissue. We recently demonstrated that in cats, the precise age at which success drops is the onset of puberty when meiotic cells first appear in the seminiferous tubules (Kim, Selvaraj *et al.* 2007).

Although xenografts from many species can produce sperm, the rate of progression of spermatogenesis varied among species. Particularly, the cat (*Felis catus*) showed a dramatic delay in producing sperm first in the study of Snedaker *et al.* (Kim, Selvaraj *et al.* 2007; Snedaker, Honaramooz *et al.* 2004), and then in our study regardless of the age of donor tissue (Kim, Selvaraj *et al.* 2007). We noted that the cat is the only carnivore to have been utilized in previous studies on testis xenografting. Because about 30% of carnivores are listed as endangered (<http://www.iucnredlist.org/>), and because concerted

efforts are needed to save these animals (due to the ecosystem-scale effects of trophic cascades that can result from loss of predators), we set out to investigate the efficacy of testis xenografting using donor tissue from other carnivore species, namely, domestic dogs (*Canis lupus familiaris*) and domestic ferrets (*Mustela putorius furo*).

Materials and Methods

Animals

Donor testes from domestic dogs were obtained from castrations at research animal facilities and local veterinary hospitals. Three donors each were used for the age groups of “Fetal” (gestation day 55-57) and “Neonatal” (1-4 weeks old), and four donors each were used for the age groups of “Prepubertal” (9-12 weeks old) and “Pubertal” (6 months old). For the ferret study, testes were obtained from domestic ferrets (Marshall BioResources, North Rose, NY) undergoing routine surgical castration under general anesthesia. Four ferrets, each aged 8 weeks, were used as donors. Testes from 18-, 28- and 31-week old ferrets were used as controls to match approximately the age of donor tissue when the xenografts were collected. All samples were stored and transported in sterile saline at 4°C, and used within 24 h after collection. The testes were washed in cold PBS and visible blood was removed by blotting. One third of each testis was fixed overnight in Bouin’s solution for histological assessment of the donor tissue at the time of xenografting. The remaining portion of each testis was processed for xenografting.

Xenografting procedure

The tunica albuginea and rete testis were removed and the testis parenchyma was cut into specimens measuring 1.5 - 2 mm³. These were kept in DMEM containing 100 µg/ml streptomycin sulfate and 100 IU/ml penicillin on ice until grafting. Male nude mice (aged 4-8 week, (Tac:Cr:(NCr)-Foxn1[nu], Taconic, Germantown, NY) were used as recipients (1-3 mice per donor). Anesthesia was induced and maintained with 1.5 - 3.5% isoflurane. Castration was performed via a midline abdominal approach, after which an incision was made on the dorsal midline and 3 - 8 xenografts were placed under the skin (2 – 4 grafts on each side, approximately 1 cm lateral of midline, and evenly spaced between the shoulders and the flanks). A 5 mm length of 6-0 silk (Ethicon, Somerville, NY) was used to mark the site of xenograft placement to facilitate retrieval, and to loosely tether the tissue to prevent movement. The dorsal incision was closed with skin staples (Braintree Scientific, Inc., Braintree, MA). At the end of surgery, buprenorphine (1 mg/kg) was used for analgesia. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University.

Analysis of xenografts

The canine testicular xenografts were collected at 10, 30, and 50 weeks after xenografting whereas the ferret xenografts were evaluated at 10, 20, 25, and 30 weeks after xenografting, corresponding to 18, 28, 33 and 38 weeks of age, respectively. At those time points, one or more larger grafts were excised from multiple recipients. The collected xenografts were measured in size and fixed in Bouin's solution overnight. The fixed xenografts were washed out of the Bouin's solution into 70% ethanol, and then dehydrated in ethanol prior to

embedding in paraffin and sectioning at 4 μm thickness. After mounting on slides, each section was deparaffinized with xylene and hydrated with 100% and 70% ethanol and water, prior to staining with hematoxylin and eosin. The sections were scanned under an Eclipse TE2000-U microscope (Nikon, Melville, NY) and images were captured using a Retiga 1300 color camera (QImaging corporation, Burnaby, BC, Canada). Statistical analysis was performed using KaleidaGraph 4 (Synergy Software, Reading, PA) and statistical significance was considered at $P < 0.05$.

Results

Histological analysis of canine xenografts

As shown in table 3.1, the recovery of xenografts decreased as the age of donor tissue approached puberty, ranging from 96 to 67%. At the time of xenografting, gonocytes were the only germ cells in the seminiferous cords of the fetal testes (Figure 3.1.A). Fetal xenografts rarely supported spermatogenesis past spermatocytes, although one xenograft did contain elongating spermatids (Figure 3.1.B and C). Testis xenografts from 3 neonatal donor dogs were placed into 3 separate recipient mice and in each case showed the same failure to support spermatogenesis (data not shown). Spermatogonia were observed as the most advanced germ cell types in all prepubertal testes prior to xenografting (Figure 3.1.D) and at 10 weeks after xenografting (Figure 3.1.E). At 30 weeks after implantation, 7.7 % of recovered xenografts had spermatocytes as the most advanced germ cell types (Figure 3.1.F). Morphologically mature spermatozoa were found in only one out of 15 grafts collected at 50 weeks (Figure 3.1.G). In the testis xenografts of pubertal dogs, post meiotic germ cells were not observed at any time points, and

spermatocytes were noted as the most advanced germ cells (Figure 3.2.I, J, and K).

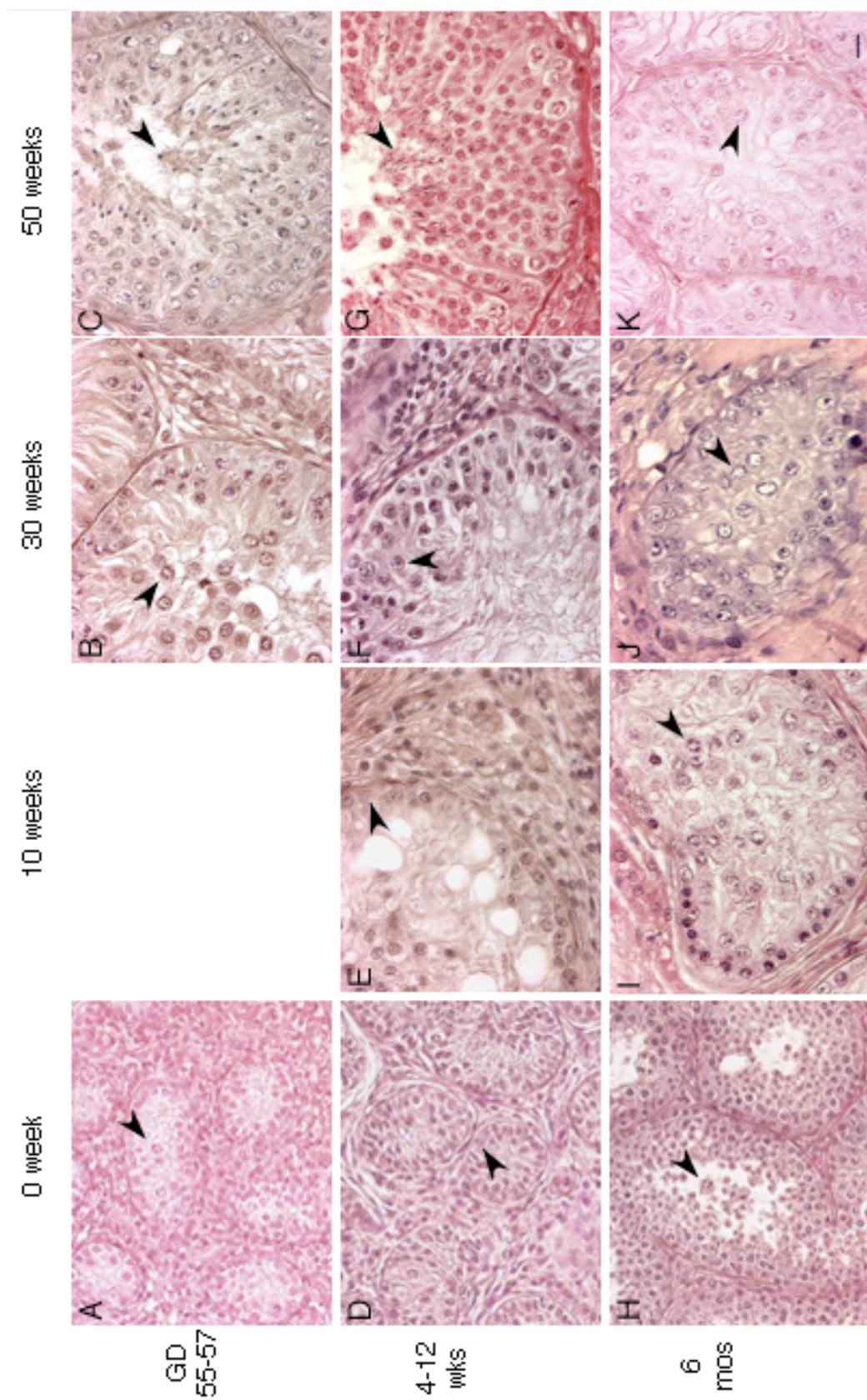
Table 3.1. Xenografts recovery and parameters of spermatogenesis in dog testis xenografts

Age	% Total grafts recovered ^a	Time after grafting (weeks)	# Grafts retrieved per time point	Most advanced germ cell stages in xenografts (%) ^b				
				SG	SC	RS	ES	Sperm
Fetal (GD 55-57)	95.7	30	11	18.2	36.4			
		50	11	18.2	36.4	-	9.1	
Prepubertal (4-12 wks)	78.3	10	6	83.3				
		30	26	19.2	7.7			
		50	15	-	66.7	-	6.7	6.7
Pubertal (6 mos)	67.1	10	17	5.9	58.8			
		30	11	-	27.3			
		50	29	3.4	41.4			

^a: Total number of xenografts retrieved divided by the total number of grafts placed

^b: Total number of xenografts which contained the indicated stage of germ cell development divided by the total number of xenografts retrieved at that time point, SG: spermatogonia, SC: spermatocytes, RS: round spermatids, ES: elongating spermatids, Sperm: spermatozoa

Figure 3.1. Cross-sections of seminiferous tubules in canine testis xenografts. Panels shown under '0 week' are representative of the histological appearance of donor tissue at the time of xenografting. Gonocytes were observed in fetal testes at gestation day 55-57 (Arrowhead in A) and spermatogonia were the most advanced germ cells in the prepubertal testes (D). Full spermatogenesis was seen in pubertal testes (H) and an arrowhead indicates a cluster of almost fully mature sperm in this panel. Xenografts were collected from the different donor age groups, fetal (GD 55-57, panels B, C), prepubertal (4-12 wks, panels E, F, G) and pubertal (6 mos, panels I, J, K), at 10, 30 and 50 weeks post grafting. Fetal xenografts were not collected at 10 week time point because the small amount of donor testis tissue limited the possible number of samples and it was too early to expect germ cell development. Spermatocytes and elongating spermatids were the most advanced germ cells in testis xenografts using fetal donor tissue at 30 and 50 weeks after xenografting, respectively (Arrowheads in B and C). Testis xenografts from prepubertal donors showed spermatogonia (E), spermatocytes (F) and sperm (G) as the most germ cell stages at 10, 30 and 50 week time points, whereas spermatocytes were the most advanced germ cells observed at any time point in testis xenografts using tissue from pubertal donors (arrowheads in I, J, and K for 10, 30 and 50 week time points, respectively). Bar = 15 μm except for panels A, D, and H (100 μm). Note that xenografts of testis tissue from all donor ages rarely supported spermatogenesis. Images in this figure represent the most successful of the xenografts for the respective times and age groups.



Analysis of ferret xenografts

A total of 32 out of 80 ferret testis xenografts (40.0%) were recovered at 10, 20, 25 or 30 weeks after placement. Primary spermatocytes and spermatozoa were the most advanced stages of germ cell development in the grafts recovered 10 and 20 weeks after placement, respectively. These corresponded exactly with the progress of germ cell development in age-matched control testes (Figure 3.2). At 25 weeks after xenografting, the xenografts continued to produce sperm but some grafts started to show fluid-distended tubules (Figure 3.3). Although spermatozoa were still present at 30 weeks, more tubules were distended and some were degenerating. Small tubular lumens with no degeneration were observed in the corresponding control testes.

Evaluation of xenograft testosterone production

Average seminal vesicle weights were 319.7 ± 91.8 , 265.0 ± 132.9 and 148.9 ± 96.5 from the recipients of fetal, prepubertal and pubertal dogs, respectively. The seminal vesicle weights of the recipients were compared to those of control mice (294.9 ± 16.3), and no significant differences were found. These data suggested that the canine testis xenografts, including those from pubertal donors, were able to produce bioactive testosterone. For the 11 recipients bearing ferret grafts, the average seminal vesicle weight was 237.7 ± 130.6 mg, which was not significantly different from controls.

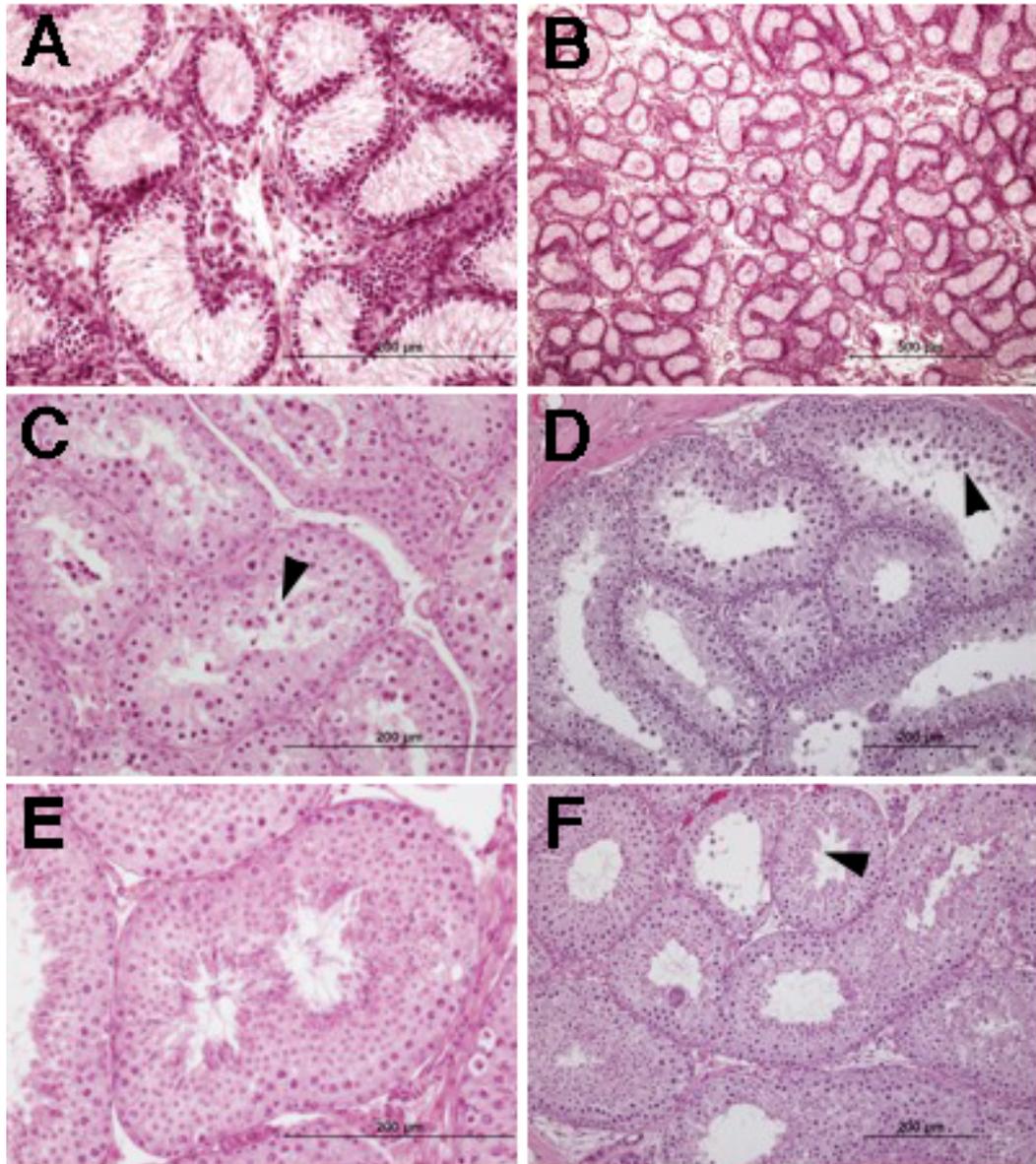


Figure 3.2. Histology of ferret seminiferous tubules in age-matched control and testis xenograft sections. No differentiating germ cells were observed in the testes collected from an 8 week old ferret (panels A and B). At 18 weeks old, spermatocytes were the most advanced germ cells in control testes (arrowhead in panel C). This stage was also the most advanced cell type observed in xenografts removed 10 weeks after placement (arrowhead in panel D). Full spermatogenesis was observed in the testis of a 28 week old ferret (E), as well as in a xenograft collected 20 weeks after placement (arrowhead in panel F).

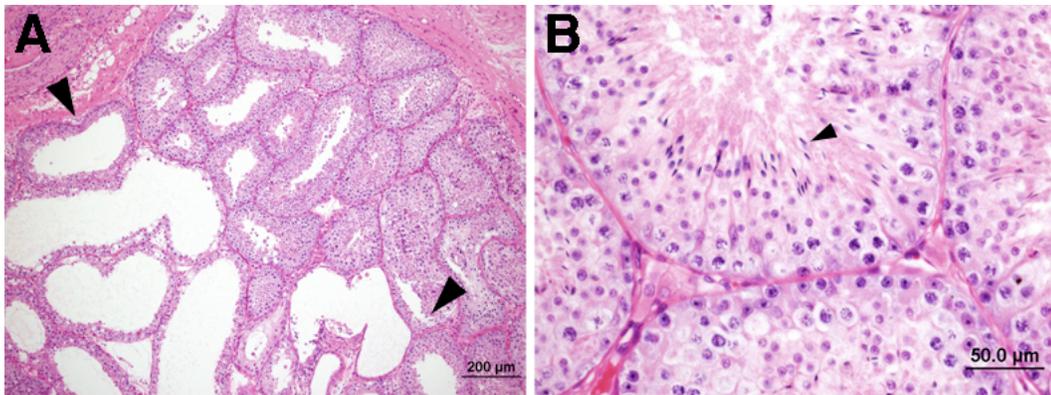


Figure 3.3. Histological sections of a ferret xenograft collected at 25-week post grafting. Some seminiferous tubules were fluid distended and showed empty lumens (the area indicated by arrowheads in panel A), whereas other tubules continued to produce sperm (arrowhead in panel B).

Discussion

In this study, we compared the success and progression of spermatogenesis in dog and ferret testis xenografts, in an effort to understand the delayed and abnormal spermatogenesis we had observed in xenografts using feline testis tissue (Kim, Selvaraj *et al.* 2007). Put simply, we wondered whether the derangements we had observed in feline xenograft spermatogenesis were unique to cats or whether they represented a broader incompatibility between the testis tissue of carnivores and the environment provided by the recipient mice. Ectopic testis xenografting has been performed using many different species as donors (Honaramooz, Snedaker *et al.* 2002; Oatley, Reeves *et al.* 2005; Rathi, Honaramooz *et al.* 2006; Schlatt, Honaramooz *et al.* 2006; Schlatt, Kim *et al.* 2002; Shinohara, Inoue *et al.* 2002; Snedaker, Honaramooz *et al.* 2004). However, there have been great discrepancies between species in terms of the timing of xenograft sperm production versus that seen under normal physiological conditions. Xenograft spermatogenesis in the pig (Honaramooz, Snedaker *et al.* 2002) and primate (Honaramooz, Li *et al.* 2004) showed a decreased time to sperm production versus if the testis tissue had been left in the donor. No difference was observed in the timing of sperm production of bovine xenografts (Oatley, de Avila *et al.* 2004; Rathi, Honaramooz *et al.* 2005), and sperm production was remarkably delayed in feline xenografts (Snedaker, Honaramooz *et al.* 2004). We also observed delayed germ cell development in cat testis xenografts, and documented that this was true regardless of donor age (Kim, Selvaraj *et al.* 2007).

In the present study, the recovery rates of canine xenografts were comparable to those we obtained for cats, 96-67% vs. 82-64%, respectively. In

contrast, the ferret xenografts showed lower total recovery rates (40%). In the dog, spermatocytes were observed as the most advanced germ cell type in neonatal (gestation day 56, which was a few days before the birth) and prepubertal (4-12 week old) xenografts at 30 week after placement, which should match to 30-42 weeks of age. Considering that the onset of puberty is 6-9 months old in the dog (Oettle 1993), the progression of spermatogenesis is similar to the intact controls and xenografts. However, the support of spermatogenesis was remarkably poor in the canine xenografts, particularly for the post meiotic cells and sperm production: only 2 and 1 grafts out of a total of 125 produced elongating spermatid and sperm, respectively.

Although the recovery rate of ferret grafts was slightly less than for other species, the progress of germ cell development in the ferret xenografts was well matched to that seen in intact control testes. In ferret xenografts, we observed spermatocytes at 10 weeks and sperm production at 20 weeks after placement. The later appearance of distended tubules is consistent with findings in the mouse, in which the tubules seem to degenerate after being productive, perhaps as a result of the lack of an outflow tract (Honaramooz, Snedaker *et al.* 2002). The production of high numbers of sperm, the apparently normal histological appearance of the productive seminiferous tubules, and the apparently normal timing of sperm production in ferret testis xenografts in comparison with controls, clearly distinguish this species from both canine and feline testis xenografts. In this regard, our study demonstrates that the difficulties seen in feline and canine xenografts are not indicative that xenografts from all carnivores would fail to support spermatogenesis. Rather, differences in success lie at either the species, genus or family levels, but are not uniform across the taxonomic order, *Carnivora*. This finding has important

practical implications for wildlife conservation and can help inform future lines of research. The factors underlying the differences in ability of testis xenografts to support spermatogenesis are not well characterized, with the exception of the marmoset (*Callithrix jacchus*), which has been shown to have an endocrinological incompatibility with the recipient mouse, in the form of a species difference in the LH receptor (Wistuba, Mundry *et al.* 2004).

Xenografts from both dogs and ferrets actively produced testosterone, based on the weight of the seminal vesicles of the recipient mice. This tissue is very androgen dependent leading to its ability to be used as a bioassay for functional testosterone levels (Rathi, Honaramooz *et al.* 2006; Schlatt, Honaramooz *et al.* 2003; Schmidt, de Avila *et al.* 2006). This proved that the interaction between host endocrine system and the Leydig cells was functionally normal. Interestingly, the dog xenografts that were recovered grew large—up to 100 times in volume—and normal tubule and interstitial cell architecture was maintained, with the exception of the lack of germ cells. This phenomenon might be explained by failure of the canine Sertoli cells to interact with the host system and to support spermatogenesis. In the dog, there are two predicted isoforms of FSH receptors, which are expressed on Sertoli cells. The protein sequence of canine FSH receptor isoform 2 is only 64% identical to the murine FSH receptor, whereas the sequence of isoform 1 is 84% identical. Further investigation is needed to confirm whether failure to respond to murine FSH is responsible for the poor spermatogenesis in the dog grafts. In conclusion, the progression of spermatogenesis and the successful production of sperm in xenografts largely depend on the nature of the donor species. Factors contributing to these species-specific responses are unknown, and could derive from the xenograft germ cells or somatic tissues,

or from the hormonal interactions between the recipient mouse and the xenografts.

In this present study, we demonstrated that the progression of spermatogenesis was similar to that seen in age-matched controls both in the dog and ferret testis xenografts but the support to produce sperm was remarkably poor in the dog xenografts. Although testis xenografting is a potentially powerful tool to preserve male genetic information, differences between species will limit the application of this technique.

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CHAPTER 4

Recipient preparation and mixed germ cell isolation for spermatogonial stem cell transplantation in domestic cats

Kim, Y., Selvaraj, V., Dobrinski, I., Lee, H., McEntee, M. C. and Travis, A. J. 2006. Recipient preparation and mixed germ cell isolation for spermatogonial stem cell transplantation in domestic cats. *J. Andrology*. 27(2):248-256

Abstract

The loss of genetic diversity poses a serious threat to the conservation of endangered species including wild felids. We are attempting to develop spermatogonial stem cell transplantation in the cat as a tool to preserve and propagate male germplasm from genetically valuable animals, be they threatened wild species or lines of cats used as models for inherited diseases. In this study, we investigated the use of local external beam radiation treatment to deplete the endogenous germ cells of male domestic cats, a step necessary to prepare them for use as recipients for transplantation. Testes of 5 month-old domestic cats were irradiated with a fractionated dose of 3 Gy per fraction for three consecutive days. These cats were castrated at 2, 4, 8, 16, and 32 weeks post-treatment, and progress of spermatogenesis was evaluated histologically and compared against age-matched controls. Even at the latest time points, less than 10% of tubules contained germ cells at any stage of meiosis, showing the efficacy of this protocol. In addition, male germ cells were isolated from the testes of domestic cats using a 2-step enzymatic dissociation to establish a protocol for the preparation of donor cells. The presence and viability of spermatogonia within this population was demonstrated by successful transplantation into, and colonization of, mouse seminiferous tubules. The success of these protocols provides a foundation to perform spermatogonial stem cell transplantation in the domestic cat.

Introduction

The irrevocable loss of genetic diversity in cats due to infertility or the death of rare individuals is a tremendous obstacle to the conservation of endangered species, as well as to the maintenance of lines of cats used to study inherited disease. To maintain the genetic information contained in individual males, spermatozoa can be collected pre- or post-mortem and cryopreserved. However, reliance on sperm alone has several limitations. For example, sperm are terminally-differentiated, haploid cells, which cannot replicate themselves and so can only be used for a limited number of breeding attempts. Notably, spermatozoa also cannot be collected from sexually immature males. Techniques of assisted reproduction based on spermatogonial stem cells (SSC) offer several advantages in this regard. Unlike mature spermatozoa, SSC replenish their own population while concurrently producing daughter cells that undergo meiosis and differentiate into sperm during the process of spermatogenesis. Even more significant for the purposes of conservation, spermatogonia can be harvested from immature males as well as adults, offering a way to preserve genes from animals that die prior to sexual maturity. In addition, the use of *in vitro* culture systems might allow for the expansion of populations of SSC (Izadyar, den Ouden *et al.* 2003a; Kubota, Avarbock *et al.* 2004), increasing potential future use of this resource.

Testis xenografting is one of the techniques that can utilize SSC to preserve valuable genetic information. This technique is performed by transplanting mm-sized cubes of testis tissue from a variety of species into immunodeficient mice, in which the xenografts can grow and produce sperm of the donor species. Xenografting of testicular tissue into mouse recipients has

been successful with tissues isolated from mice, pigs, and goats (Honaramooz, Snedaker *et al.* 2002), hamster and monkey (Schlatt, Kim *et al.* 2002), calves (Oatley, Reeves *et al.* 2005), rabbits (Shinohara, Inoue *et al.* 2002), and cats (Snedaker, Honaramooz *et al.* 2004). Although testis xenografting is relatively easy to perform technically and requires only immunodeficient mice regardless of the donor species, this method also has its own set of limitations. For example, it takes a full year for feline sperm to be produced from xenografted testis tissue (Snedaker, Honaramooz *et al.* 2004), and even if successful, the method provides relatively low numbers of donor spermatozoa. Because the sperm produced are testicular and have not undergone epididymal maturation, they can only be used for intra-cytoplasmic sperm injection (ICSI) followed by embryo transfer. From a conservation perspective, this is limiting in that it requires the development of both these technologies for all species in which it could be used. In addition, the relatively short lifespan of immunodeficient mice would limit the time available to retrieve xenograft-derived sperm. This would necessitate the development of cryopreservation methods for long-term storage of testis tissue cubes that preserve the multiple cell types and tissue architecture, a feat more difficult than the cryopreservation of single cell suspensions.

Spermatogonial stem cell transplantation (SSCT) in the mouse was first reported in 1994 (Brinster and Zimmermann 1994). In this technique, either enriched populations of spermatogonia, or mixed cell populations including spermatogonia, are placed within the lumens of the seminiferous tubules of a recipient. Placement is performed either by retrograde injection through the efferent ducts [rodents; (Ogawa, Arechaga *et al.* 1997)], or via retrograde injection into the rete testis [large animal models; (Honaramooz,

Behboodi *et al.* 2003; Honaramooz, Megee *et al.* 2002). This technique has several advantages over testis xenografting. Namely, there is the potential for increased numbers of sperm to be collected and these sperm will undergo epididymal maturation, both of which might allow the sperm produced to be used for other technologies of assisted reproduction such as in vitro fertilization or artificial insemination. In addition, depending upon the species of recipient, sperm could be collected via electro-ejaculation or by use of manual stimulation or artificial vaginas over a period of time longer than the lifespan of a rodent. SSCT could allow sperm collection over a time period covering multiple estrus cycles and give more attempts to generate offspring carrying that male's genetic information. Xenogeneic SSCT, in which the donor and recipient are different species, has been performed using several species as donors and mice as recipients (Clouthier, Avarbock *et al.* 1996; Ogawa, Dobrinski *et al.* 1999a). However, if the phylogenetic distance between donor and recipient is too wide, the donor spermatogonia can colonize but spermatogenesis will not occur (Dobrinski, Avarbock *et al.* 1999; Nagano, McCarrey *et al.* 2001). Therefore, we sought to investigate methods that would allow the use of SSCT in felids, for the purpose of preserving the genetic diversity of genetically valuable cats.

To perform SSCT in different species of animals, two distinct steps must be achieved prior to the actual introduction of donor germ cells. First, a recipient animal should have its endogenous germ cells depleted, so that the introduced cells will have improved access to the basal compartment of seminiferous tubules and so that there is a higher relative yield of donor-derived sperm (Brinster, Ryu *et al.* 2003). Several techniques have been used to reduce or deplete endogenous male germ cells such as irradiation

(Meistrich, Hunter *et al.* 1978; Van Beek, Meistrich *et al.* 1990; Withers, Hunter *et al.* 1974), chemotherapeutic drugs (Brinster, Ryu *et al.* 2003; Ogawa, Arechaga *et al.* 1997) and cold ischemia treatment (Young, Goldstein *et al.* 1988). External beam radiation treatment is a useful tool in this regard, because the germ cells are highly radiosensitive (Dym and Clermont 1970; Huckins 1978), and the Sertoli cells and Leydig cells are relatively radioresistant (Dym and Clermont 1970; Joshi, Yick *et al.* 1990; van der Meer, Huiskamp *et al.* 1992; Vergouwen, Huiskamp *et al.* 1994). A number of radiation treatment protocols have therefore been tested and used to prepare recipients of several species for SSCT (Izadyar, den Ouden *et al.* 2003b; Schlatt, Foppiani *et al.* 2002). Second, once having prepared the recipient, a cell suspension containing spermatogonia must be isolated from donor testes. Optimally, mixed germ cell populations would be enriched in spermatogonia (Shinohara and Brinster 2000; Shinohara, Orwig *et al.* 2000), although the lack of stem cell markers in species other than rodents and primates makes such a step premature at this time. Spermatogonia are situated in the basal compartment of seminiferous tubules, located between Sertoli cells and just above the underlying basement membrane and peritubular myoid cells. In the interstitial space between tubules, blood and lymphatic vessels, connective tissue and Leydig cells are positioned. Collection of suspensions of individualized male germ cells has commonly utilized sequential enzymatic digestion (Bellve, Millette *et al.* 1977; Honaramooz, Megee *et al.* 2002). Such protocols typically involve isolation of seminiferous tubules from the interstitial tissue, and then dissociation of individual germ cells from within the tubules. Although germ cell dissociation has been performed in several species, it has not yet been reported in domestic cats.

In this study, we report successful protocols for the depletion of endogenous germ cells in domestic cats by local external beam radiation treatment and for the enzymatic dissociation of testis tissue to yield mixed male germ cells containing viable spermatogonia, two critical steps necessary to perform SSCT in felids.

Materials and Methods

Reagents

All reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted. Ketamine HCl (Fort Dodge Laboratories Inc, Fort Dodge, IO), diazepam and isoflurane (Abbott Laboratories, North Chicago, IL) were used for the induction and maintenance of anesthesia in the experimental cats. For histology, 100% and 70% ethanol (Pharmco, Brookfield, CT), hematoxylin, and eosin (Electron Microscopy Sciences, Fort Washington, CA) were used. Trypan blue was purchased from Gibco (Carlsbad, CA) for cell viability analysis.

Animals

Domestic short haired cats (*Felis domesticus*), were obtained from Liberty Research Inc. (Waverly, NY). The cats were housed in groups of four under standard lighting (12L:12D) and allowed access to food and water *ad libitum*. NCr Swiss nude (nu/nu) mice aged 10-20 weeks were used as recipients for transplantation (Taconic, Germantown, NY). All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committees of Cornell University or the University of Pennsylvania.

Local external beam radiation of testes

5 month-old domestic cats (n=10) were anesthetized with ketamine HCl (5mg/kg i.v.) and diazepam (0.5 mg/kg i.v.) and maintained by masking with isoflurane (1 - 3 %). Each cat was positioned in sternal recumbency using a vacuum cushion (Vac-Lok,TM Med-Tec, Orange City, IO). The hind limbs were extended caudo-laterally to facilitate alignment of the long axes of the testes parallel to the body axis. Gauze bandage was loosely tied around the base of the scrotum to effect partial immobilization of the testes in the scrotum during the procedure. Tissue equivalent material was placed around the scrotum to protect surrounding tissue, and 1 cm of tissue equivalent material was placed on the surface of the scrotum to provide uniformity of radiation dose delivery (Figure 4.1). The testes were irradiated with a 6 MV linear accelerator (7 MeV electrons, dose rate of 300 MU/minute). A 3 cm diameter cone was used to collimate the electron beam to irradiate both testes while minimizing exposure of surrounding normal tissue. A fractionated dose of 3 Gy was applied to the testes daily for three consecutive days for a total dose of 9 Gy per animal. As discussed below, this treatment regimen was based on findings in other species that suggested fractionated protocols to be more efficacious than those involving just one exposure.

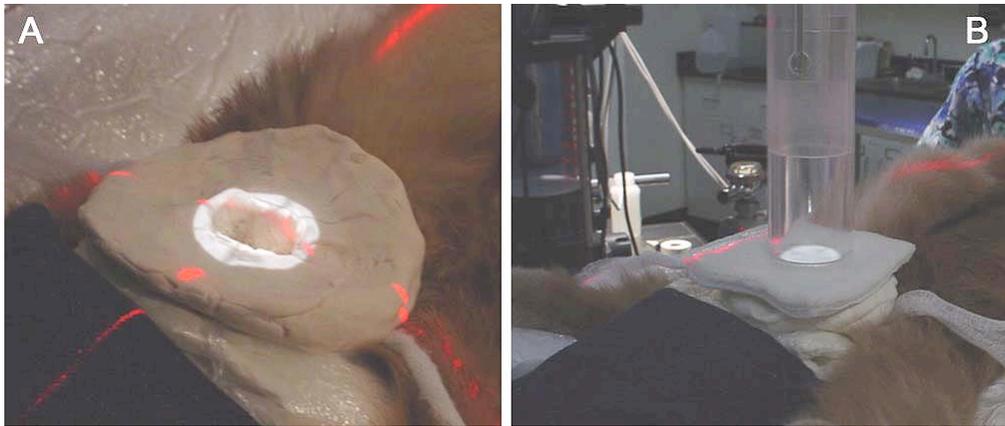


Figure 4.1. Positioning of the testes for external beam radiation treatment. The cat was positioned on a vacuum cushion repositioning device and the beam was centered precisely between the two testes (panel A). Panel B shows the collimating cone atop the tissue equivalent material.

Testis collection and processing

Cats were castrated after induction of anesthesia as described above and testes along with the epididymides were collected at time points 2, 4, 8, 16, and 32 weeks after treatment (n=2 for each time point). The testes were halved along the longitudinal axis and fixed in Bouins' solution. Testes were also obtained from routine castrations of untreated cats at local veterinary hospitals. Age-matched specimens served as controls and were processed identically as the treated testes.

Detection of spermatozoa

The cauda epididymides obtained from the irradiated animals and from the age-matched controls were minced and incubated in PBS (pH 7.4) at 37°C for 15 minutes to swim out spermatozoa. Collected sperm was observed under a light microscope at 100X magnification to evaluate their presence, appearance, and concentration. Total numbers of sperm were then calculated by multiplying the concentration by the total volume. Comparisons of epididymal sperm numbers were performed by an unpaired Student's t-test (Origin 7.0 Software, OriginLab Corporation, Northampton, MA). Statistical significance was assumed at $P < 0.05$.

Testis histology and evaluation

The fixed testes were washed out of the Bouins' solution into 70% ethanol, then dehydrated in ethanol prior to embedding in paraffin and sectioning at 4 mm. After mounting on slides, each section was deparaffinized and hydrated with xylene, and then 100% and 70% ethanol, prior to staining with hematoxylin and eosin. The sections were scored for the presence of

meiotic cells and stage of spermatogenesis in at least 500 seminiferous tubule cross sections per testis. Then the percents of tubules containing spermatocytes, round spermatids, and elongating spermatids were calculated. The results were compared with those obtained from age-matched, untreated controls. Images were captured using an Eclipse TE2000-U microscope (Nikon, Melville, NY) and Retiga 1300 color camera (QImaging corporation, Burnaby, BC, Canada).

Germ cell dissociation

Spermatogenic cells were collected from testes obtained from routine castrations of pre-pubertal, pubertal and young adult animals at a local veterinary hospital and animal shelters. A two-step enzymatic digestion was performed, as described by Dobrinski et al (Dobrinski, Avarbock *et al.* 1999). Hank's Balanced Salt Solution (HBSS) containing 0.44 mM KH_2PO_4 , 137 mM NaCl, 5.36 mM KCl, 4.2 mM NaHCO_3 , 0.44 mM KH_2PO_4 and 5 mM glucose was prepared and sterilized by passing through a 0.22 mm filter (Millipore, Billerica, MA). After rinsing the testis in this medium, visible blood was blotted and the testis was rinsed again. The tunica albuginea and grossly visible connective tissue associated with the rete testis were then removed. The remaining tissue was incubated in HBSS containing 1 mg/ml collagenase for 10 minutes at 34 °C in a shaking water bath set at 110 oscillations/min. The dispersed seminiferous tubules were isolated by allowing them to sediment in HBSS on ice and decanting the supernatant. This step was repeated until the supernatant was clear. The isolated seminiferous tubules were then incubated in HBSS containing 1.25 mg/ml trypsin and DNaseI (50 mg/ml) as above. The resultant cell suspension was filtered through a 70 mm nylon mesh (BD Falcon,

San Jose, CA), washed by centrifugation at 600 x g for 5 minutes at room temperature for three times, and resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 100 mg/ml streptomycin sulfate and 100 IU/ml penicillin. Cell viability was analyzed by incubation with 0.4 % trypan blue for 10 min at 37 °C.

Assessment of the presence and viability of SSC isolated during germ cell dissociation

The individualized germ cell population produced by the dissociation protocol was examined for the presence of viable SSC by methods similar to those published previously (Dobrinski, Avarbock *et al.* 1999). Briefly, a suspension of individual germ cells was prepared as above. Twenty NCr Swiss nude mice were treated with busulfan (40 mg/kg) to deplete endogenous male germ cells. Transplantation of the individualized male germ cells into the seminiferous tubules of these testes involved a retrograde injection through the efferent ducts of approximately 10 μ l of cell suspension (10^8 cells/ml). To analyze the success of transplantation, recipient testes were collected between 48 and 456 days post-transplantation. The tunica albuginea was removed, and the seminiferous tubules were gently dispersed with collagenase prior to fixation in freshly-prepared 4% paraformaldehyde for 2 h at 4°C. Whole-mount immunohistochemistry using a polyclonal antibody against cat testicular cells [prepared and purified as described for rabbit testis-specific and dog testis-specific antisera (Dobrinski, Avarbock *et al.* 1999)] was performed to detect the presence of feline cells in the recipient mouse testes. Cells were visualized with 3-amino-9-ethylcarbazole following incubation with biotinylated, species-specific IgG and avidin coupled to horseradish

peroxidase (Dobrinski, Avarbock *et al.* 1999). Controls included immunohistochemistry in the absence of the primary antiserum (control for specificity of detection), and immunohistochemistry of tubules soon after injection (positive control).

Results

Evaluation of epididymal spermatozoa

Only rare, immotile epididymal sperm were observed at 2 weeks after treatment. No sperm were found in the cauda epididymides of castrated testes at 4, 8, and 16 weeks after external beam radiation treatment, whereas epididymal sperm were seen in all controls at those times. At 32 weeks after treatment, sperm were collected from cauda epididymides of treated animals, as well as controls. The average total number of sperm collected from both epididymides of treated testes at 32 weeks was $3.27 \pm 1.7 \times 10^6$ (average, n = 2), significantly lower than the average total number of sperm collected from age-matched testes which was $7.73 \pm 0.4 \times 10^7$ (average, n = 2).

Effect of external beam radiation on male germ cell development

The rationale behind the choice of the parameters of the external beam radiation protocol, as well as the choice of age of the cats at time of treatment, are discussed below. At 2 and 4 weeks after treatment, <1.5% of seminiferous tubules contained meiotic cells, compared with >50% of tubule cross sections in age-matched controls (Table 4.1). In addition, most cross sections of tubules showed disarranged Sertoli cells (Figure 4.2.A and 4.2.C). At 8, 16, and 32 weeks post-treatment, 4 – 9 % of seminiferous tubules contained spermatocytes, whereas full spermatogenesis was seen in all tubules in age-

Table 4.1.
Progression of spermatogenesis in treated and untreated, control testes

Time after treatment (weeks)	% of tubule cross sections containing:				% of tubule cross sections in age-matched controls containing:			
	SC*	RS*	ES*	Sperm	SC	RS	ES	Sperm
2	1.4 ± 1.58	1 ± 3.16	0.1 ± 0.37	0 ± 0	37 ± 4.06	11.6 ± 8.01	0 ± 0	0 ± 0
4	0.4 ± 0.84	0.4 ± 0.52	0.5 ± 0.71	0 ± 0	62.8 ± 6.45	55 ± 6.51	49.2 ± 4.76	15.8 ± 3.83
8	8.9 ± 4.25	1.1 ± 1.20	0.4 ± 0.52	0 ± 0	68.2 ± 5.72	41.6 ± 10.64	48.4 ± 6.42	13.6 ± 2.30
16	5.7 ± 5.51	5.6 ± 2.79	2.9 ± 2.81	0 ± 0	71.2 ± 3.83	59.4 ± 5.41	43.6 ± 8.53	19.8 ± 4.09
32	4.1 ± 4.91	1.6 ± 2.11	1.9 ± 2.45	0.7 ± 1.33	74.6 ± 7.64	53.2 ± 5.89	50.2 ± 2.39	29.2 ± 7.66

*SC: spermatocytes, RS: round spermatids, ES: elongating spermatids

Two treated animals and one untreated control animal were examined for each time point. Values are expressed as the mean percentage of tubule cross-sections showing a given stage of spermatogenesis, ± the standard deviation. Values were generated by counting 100 tubule cross-sections within 5, non-sequential histological sections of each testis, and generating mean and standard deviation using Origin 7.0 software.

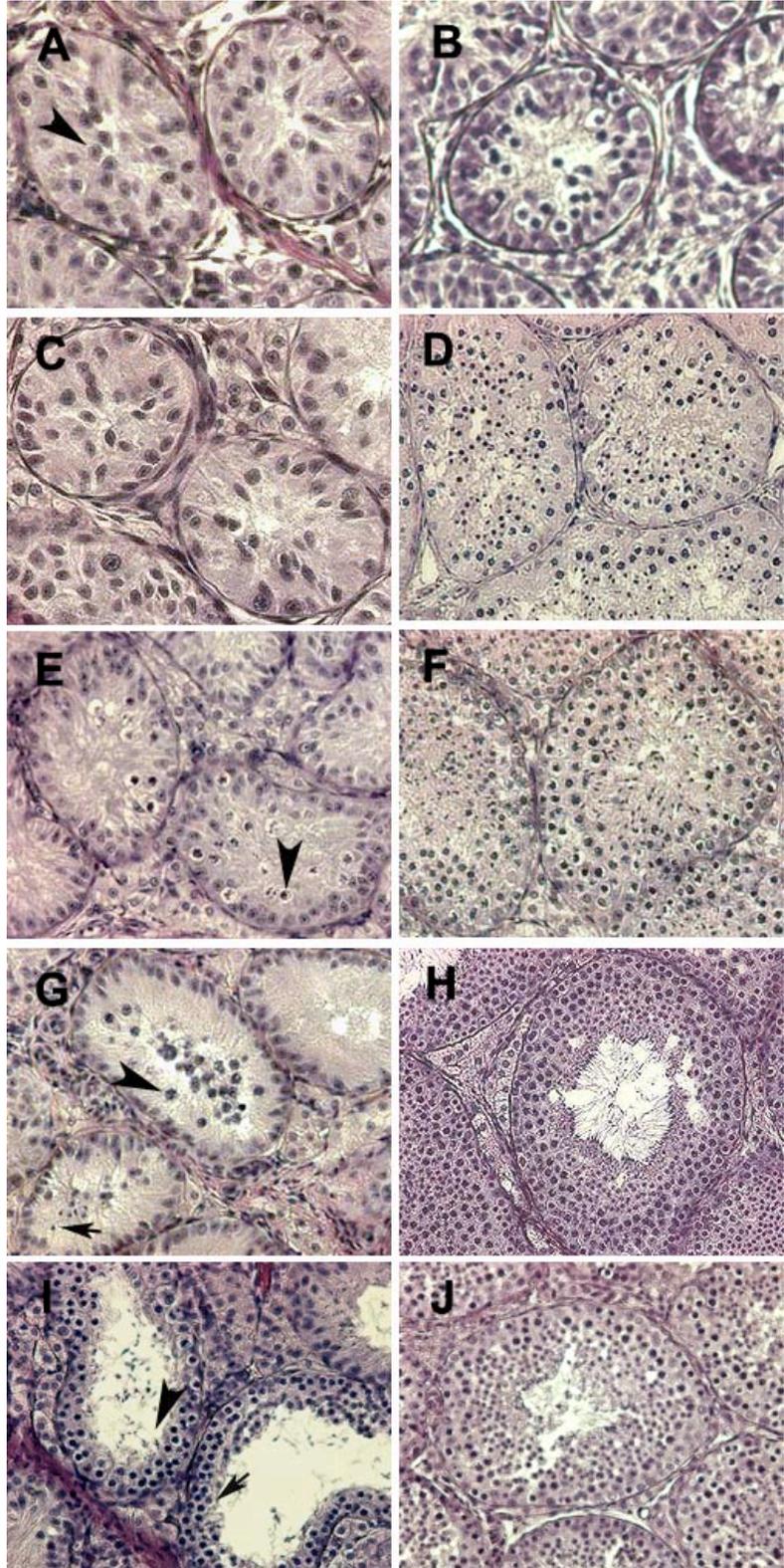
matched controls (Table 4.1 and Figure 4.2). At 32 weeks after treatment, a very small number of tubules (< 1%) had sperm, compared with age-matched controls (Figure 4.2.I and 4.2.J). There was no apparent change in the interstitial cell population between irradiated and untreated testes (Figure 4.2).

Isolation of mixed germ cells

Successful mixed germ cell isolation in the cat relied upon protocols more similar to those used in large animal models than rodents, due to the density of the connective tissue within the tunica albuginea. Removal of the testis parenchyma from this capsule and from connective tissue associated with the rete was performed by sharp dissection. This decreased the time of exposure to collagenase and resulted in less damage to the seminiferous tubules. Similar to male germ cell dissociation from rodent testes, collagenase treatment largely removed interstitial Leydig cells, endothelial cells, and blood cells. The seminiferous epithelium was then dissociated into a suspension of single cells when incubated with 1.25 mg/ml trypsin. After washing, the resultant mixed cell suspension included spermatogonia, spermatocytes, and round and elongating spermatids (data not shown). This protocol was used successfully on testes from pre-pubertal, pubertal, and adult cats.

Approximately 90% of the cells excluded trypan blue, suggesting viability. Because of the large inter-cellular bridges connecting male germ cells (Ravindranath, Dettin *et al.* 2003), spermatids often coalesced into larger, multi-nucleated cells (data not shown). This phenomenon is also seen during the separation of murine male germ cells (Bellve, Cavicchia *et al.* 1977).

Figure 4.2. Histological appearance of irradiated and age-matched, untreated control testes. Irradiated testis tissue collected at 2 (panel A; the arrow head points to a Sertoli cell), 4 (panel C), 8 (panel E; the arrow head points to a spermatocyte), 16 (panel G; the arrow head points to a spermatocyte, and the arrow to an elongating spermatid) and 32 (panel I; the arrow points to a spermatocyte, and the arrowhead to spermatozoa) weeks after irradiation. Untreated control, age-matched testis tissue corresponding to 2 (panel B), 4 (panel D), 8 (panel F), 16 (panel H) and 32 (panel J) weeks after irradiation (200X).



Assessment of the presence and viability of SSC's isolated during germ cell dissociation

To demonstrate that viable SSC's were contained within the mixed cell population produced by the dissociation procedure, the cell suspension was injected via the efferent ductules into the seminiferous tubules of germ cell-depleted mice. As has been found with other xenogeneic SSCT trials (Dobrinski, Avarbock *et al.* 1999), the feline SSC were able to colonize the murine seminiferous tubules but the environment within them was not supportive of feline spermatogenesis. Using whole mount immunohistochemistry, 19 of 20 recipients stained positive with an antiserum that recognized feline testicular cells, and this staining was present in recipients throughout the time period observed (Figure 4.3).



Figure 4.3. Detection of cat testis cells in mouse seminiferous tubules. Injected feline testicular germ cells colonized mouse seminiferous tubules and stained with an antibody specific for cat testicular cells in whole-mount immunohistochemistry on 63 days (panel A, 16X), 120 days (panel B, 40X) and 430 days (panel C, 16X) after transplantation. The negative control of tubules from testes receiving transplanted feline germ cells but incubated in the absence of primary antibody revealed no staining (data not shown).

Discussion

SSCT offers a powerful complement to the collection of mature spermatozoa in efforts to preserve the breeding potential of males. These benefits are based on the fact that spermatogonia are stem cells and can therefore replenish their own population, while simultaneously producing sperm on a renewable basis. This technique has been performed using SSC from rodents (Clouthier, Avarbock *et al.* 1996; Ogawa, Arechaga *et al.* 1997; Ogawa, Dobrinski *et al.* 1999a), large domestic animals (Dobrinski, Avarbock *et al.* 2000; Honaramooz, Behboodi *et al.* 2003; Honaramooz, Megee *et al.* 2002; Izadyar, den Ouden *et al.* 2003b), dogs and rabbits (Dobrinski, Avarbock *et al.* 1999), and primates (Nagano, McCarrey *et al.* 2001). To make the technique effective for the preservation of threatened or endangered felids, preparation of recipient testes and donor germ cells in a suitable model is crucial.

The first step necessary for SSCT is the preparation of the recipient testis, which involves a reduction of the endogenous SSC population. This has two purposes: it increases the success of colonization by opening up the appropriate "niches" for the transplanted SSC (Spradling, Drummond-Barbosa *et al.* 2001), and it improves the relative yield of donor-derived versus recipient-derived sperm. To accomplish this, we opted to use fractionated external beam radiation treatment, which has been demonstrated to be an efficient method for germ cell depletion, while also avoiding the complications of systemic drug treatments such as busulfan, a DNA alkylating agent that destroys proliferating cells. Busulfan can therefore affect proliferating cells elsewhere in the body, such as in the bone marrow, as well as endogenous germ cells (Ogawa, Dobrinski *et al.* 1999b). The testis has been characterized

as having radiosensitive cells (the male germ cells, especially spermatogonia) and radioresistant cells (the supporting somatic cells) (Dym and Clermont 1970; Huckins 1978; Joshi, Yick *et al.* 1990; van der Meer, Huiskamp *et al.* 1992; Vergouwen, Huiskamp *et al.* 1994). In the human, very low doses (<0.35 Gy) result in a sometimes transitory depletion of spermatogenesis, low doses (<2 Gy) affect primarily germ cells but on a more permanent basis, and doses in excess of 20 Gy begin to affect Leydig cells (Shalet 1993). In bovine calves, a single dose of 10-14 Gy was sufficient to eliminate spermatogenesis in 60% of the tubules (Izadyar, den Ouden *et al.* 2003b). When pre-pubertal rats were irradiated with a single dose of 3 Gy, all the research animals showed a resumption of endogenous spermatogenesis by 70 days post-treatment (Guitton, Touzalin *et al.* 2000). A two-day, fractionated ionizing radiation protocol was described for use in mice, in which a dose of 1.5 Gy was followed by a dose of 12 Gy. At 21 weeks post-treatment, endogenous spermatogenesis was reduced to <10% of normal levels, suggesting that fractionated radiation protocols might improve duration of effect (Creemers, Meng *et al.* 2002). These varied results show that species, dosage and regimen, and the age of the subject might have significant effects on the long-term outcome of treatment. No large-scale studies comparing the effects of these variables on the efficacy of different irradiation protocols have been performed. The protocol reported herein for the cat was therefore devised to have a focal as opposed to systemic effect and to utilize a fractionated low-dose regimen as most likely having a longer-term effect. The protocol was performed at a dose consistent with previous successful reports in the literature. Our results demonstrated that when administered to the testes of 5 month-old domestic cats, this fractionated protocol of 3 Gy/day for three

consecutive days successfully depleted endogenous male germ cells. We chose this age for technical reasons, including that the testes at that age are of sufficient size to manipulate for external beam radiation as well as being sized appropriately for any subsequent transplantation procedures. Older cats were not used in the irradiation study for several reasons. First, the presence and subsequent death of higher numbers of germ cells might increase the time required until a transplant could be performed successfully. Transplantation will not be performed immediately after irradiation to give the Sertoli cells the opportunity to remove dead germ cells, and increase access to stem cell niches along the basement membrane. Second, if transplantation were to be performed for the purpose of breeding with donor-derived sperm, then there would be a desire to have as long a lifespan as possible post procedure so that sperm could be collected over the time period required.

Spermatogenesis in domestic cats usually begins when 5 or 6 months old (Tsutsui, Kuwabara *et al.* 2004), and the spermatogenic cycle takes 46.8 days (Franca and Godinho 2003). At 2 weeks post-treatment, rare sperm were collected from the epididymides, suggesting that spermatogenesis had begun in isolated areas of individual tubules before treatment, sometime during their fourth month. No epididymal sperm were found from the irradiated testes at 4, 8, and 16 weeks after treatment. Sperm were collected from the irradiated animals at 32 weeks after treatment; however, the number of sperm was over 20-fold less than epididymal sperm of normal young adult cats. These results showed that the current radiation protocol depleted most of the spermatogonia but didn't destroy the ability of those that remained to complete spermatogenesis, nor did it destroy the ability of the Leydig cell and Sertoli cell populations to support spermatogenesis. Our observations of the tubules in

cross section supported this conclusion. Despite the loss of any meiotic cells and most SSC, and the disorganization of Sertoli cells seen at 2 and 4 weeks after treatment, reorganization of the architecture of the seminiferous epithelium occurred between 4-8 weeks, and spermatocytes began to be observed by week 8. The timing of this recovery suggests that approximately 4-8 weeks post-treatment would be optimal for SSCT.

Prior to transplantation, donor SSC must be separated from other cells within the testis. It is optimal to transplant populations of mixed germ cells enriched in SSC (Shinohara and Brinster 2000; Shinohara, Orwig *et al.* 2000). However, given the absence of any known cell surface markers for SSC in cats, we sought to begin by separating mixed populations of feline male germ cells from testicular somatic cells. Protocols for the preparation of isolated male germ cells are species-specific because each species has its own anatomical characteristics, such as the relative amounts of connective tissue between tubules, lobulation, and the ease of removing the tunica. For this reason, we compared two protocols, those of Bellvé *et al.* (Bellvé, Millette *et al.* 1977) and Dobrinski *et al.* (Dobrinski, Avarbock *et al.* 1999), the latter of which proved more efficient for domestic cats. Before exposure to digestive enzymes, two mechanical steps were required: removal of testicular vessels reduced contamination with blood, and removal of the testicular capsule and grossly visible connective tissue associated with the rete testis facilitated a more uniform digestion of the testicular parenchyma. A sequential enzymatic digestion was then used to individualize a population of mixed cells. As in the mouse, treatment with trypsin led to the loss of developing flagella in elongating spermatids and the appearance of some multi-nucleated round spermatids. With this protocol, we obtained mixed germ cells with minimal

visual contamination of blood cells and interstitial cells. In the future, the enrichment of SSCs within this mixed germ cell milieu will be pursued once SSC surface markers are identified in felids.

The presence of viable SSC within this population was demonstrated by the successful colonization of feline cells within murine seminiferous tubules, although as with other donor species, the environment within the murine seminiferous epithelium did not support feline spermatogenesis. Because there is no antibody specific for cat spermatogonial stem cells, we utilized an antiserum made against feline testicular cells to recognize cells of feline origin within the murine seminiferous tubules. In 19/20 recipient mice, cells staining positive were found. Typically, the immunoreactive cells were single or arranged in small groups along the basement membrane, indicating colonization and initial proliferation of feline type A spermatogonia in the mouse seminiferous tubules. These data verified the viability of feline SSC within the dissociated cell population. Together with the demonstration of a successful irradiation protocol for depletion of endogenous male germ cells in the cat testis, these data provide a foundation upon which to perform spermatogonial stem cell transplantation in the feline model system.

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CHAPTER 5

Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog

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Abstract

Spermatogonial stem cell transplantation offers unique approaches to investigate spermatogonial stem cells (SSC) and to manipulate the male germline. We report here the first successful performance of this technique in the dog, which is an important model of human diseases. First, we investigated an irradiation protocol to deplete endogenous male germ cells in recipient testes. Histologic examination confirmed >95% depletion of endogenous SSC, but retention of normal testis architecture. Then, five month-old recipients (n=5) were focally irradiated on their testes prior to transplantation with mixed seminiferous tubule cells [fresh (n=2) or after two weeks of culture (n=3)]. The dogs receiving cultured cells showed an immediate allergic response, which subsided quickly with palliative treatment. No such response was seen in the dogs receiving fresh cells, for which a different medium was used. 12 months post injection, recipients were castrated and sperm was collected from epididymides. We performed microsatellite analysis comparing DNA from the epididymal sperm with genomic DNA from both the recipients and the donors. We used 6 markers to demonstrate the presence of donor alleles in the sperm from one recipient of fresh mixed tubule cells. No evidence of donor alleles was detected in sperm from the other recipients. Using quantitative PCR based on single nucleotide polymorphisms, about 19.5% of sperm were shown to be donor-derived in the recipient. Our results demonstrate the first successful completion of SSCT in the dog, an important step toward transgenesis through the male germline in this valuable biomedical model.

Introduction

Spermatogonial stem cell transplantation (SSCT) was first reported in the mouse (Brinster and Zimmermann 1994). In this technique, mixed germ cell populations containing spermatogonia, often enriched to some degree, are placed within the lumens of the seminiferous tubules of a recipient. Since the first report, isolated germ cells from various donor species including rat (Clouthier, Avarbock *et al.* 1996; Ogawa, Arechaga *et al.* 1997), hamster (Ogawa, Dobrinski *et al.* 1999a), rabbit and dog (Dobrinski, Avarbock *et al.* 1999), primate (Nagano, McCarrey *et al.* 2001), bull (Oatley, de Avila *et al.* 2002) and cat (Kim, Selvaraj *et al.* 2006) have been transplanted into mouse testes. Rats and hamsters were able to produce sperm in the recipient mouse testes, whereas the other species showed colonization of stem cells but not spermatogenesis. These studies showed that the phylogenetic distance between recipients and donors was a strong determinant of whether the recipient environment would support donor spermatogenesis.

Allogeneic transplantation has been performed in the goat (Honaramooz, Behboodi *et al.* 2003; Honaramooz, Megee *et al.* 2007) and pig (Honaramooz, Megee *et al.* 2002). Such transplantation between con-specific individuals is desired for many practical and experimental purposes. In addition, transplantation between species might have value for the conservation of endangered wildlife. Optimization of SSCT in diverse species requires several steps. In addition to a close phylogenetic relationship with the donor, an ideal recipient would have its endogenous germ cells depleted. This would give introduced SSC improved access to the basal compartment of seminiferous tubules, allowing more room for colonization and expansion

within the stem cell niche, and would ultimately result in a higher relative yield of donor-derived sperm (Brinster, Ryu *et al.* 2003).

Two techniques have been widely used to reduce or deplete endogenous male germ cells in recipients: focal irradiation (Izadyar, den Ouden *et al.* 2003; Schlatt, Foppiani *et al.* 2002) and chemotherapeutic drugs (Brinster, Ryu *et al.* 2003; Ogawa, Arechaga *et al.* 1997). In non-rodent animals, focal irradiation can provide an advantage of not inducing systemic effects in the recipient, whereas the systemic effects of chemotherapeutics will vary between species, as well as with the nature of the drug, its dose, and route of administration. Once the recipient testis has been prepared, transplantation is performed either via retrograde injection through the efferent ducts [rodents (Ogawa, Arechaga *et al.* 1997)], or into the rete testis [large animal models (Herrid, Vignarajan *et al.* 2006; Honaramooz, Behboodi *et al.* 2003; Honaramooz, Megee *et al.* 2002; Izadyar, den Ouden *et al.* 2003)]. An additional concern in SSCT is the potential for rejection of the introduced cells by the recipient's immune system. To overcome this problem, matching the strains of donors and recipients was addressed in mice (Brinster and Zimmermann 1994). In xenogeneic transplantation, in which cells from different donor species were transplanted into mice, immunodeficient mice were used to avoid rejection (Clouthier, Avarbock *et al.* 1996; Dobrinski, Avarbock *et al.* 1999; Dobrinski, Avarbock *et al.* 2000; Ogawa, Dobrinski *et al.* 1999b). However, in several species heterologous transplantation between individuals within the same species has been shown to be successful even in the absence of modulation of the recipient's immune system (Herrid, Vignarajan *et al.* 2006; Honaramooz, Behboodi *et al.* 2003; Honaramooz,

Megee *et al.* 2002). Thus, several aspects of this technology require species-specific testing and optimization.

Stem cell-based technologies such as SSCT potentially offer both clinical and basic scientific applications. Transplantation of SSC from wild-type mice into the testes of mice having genetic infertility showed successful restoration of spermatogenesis in the recipient testes (Rilianawati, Speed *et al.* 2003). Thus SSCT has been suggested to be useful to preserve fertility in both human and non-human animals, such as for human patients receiving chemotherapy (Fujita, Ohta *et al.* 2005; Fujita, Tsujimura *et al.* 2006) or wildlife conservation (Pukazhenthil, Comizzoli *et al.* 2006). From a basic scientific perspective, SSCT can be used to investigate the fundamental characteristics of SSC (Kent Hamra, Chapman *et al.* 2005; Kubota, Avarbock *et al.* 2003; Nagano, Avarbock *et al.* 1999; Parreira, Ogawa *et al.* 1998). Furthermore, because the male germline can be manipulated in the SSC prior to transplantation, SSCT can be used to generate transgenic animals (Honaramooz, Megee *et al.* 2007; Kanatsu-Shinohara, Ikawa *et al.* 2006; Nagano, Brinster *et al.* 2001; Ryu, Orwig *et al.* 2006).

Over 360 naturally-occurring canine genetic diseases have been shown to have counterparts in humans, including various forms of cancer (Lingaas, Comstock *et al.* 2003), blindness (Acland, Aguirre *et al.* 2005), and orthopedic defects (Athanasίου, Agarwal *et al.* 1995). Moreover, diseases such as muscular dystrophy and bleeding disorders (Tsai, Clark *et al.* 2007) have been shown to involve the same genes in both species. More than 400 different dog breeds have been produced by selective breeding, many of which originate from only a few founders and/or have undergone population bottlenecks. Many purebred dogs have unique phenotypic traits including susceptibility to certain

genetic diseases. Thus the dog is an outstanding model in which linkage analysis can be used to identify genes as candidates for causing a specific phenotype. Development of canine transgenesis will provide new opportunities for verification that identified genes are actually causative of such phenotypes (e.g. when that gene is placed in a different genetic background), and for studying mechanisms that cause, and developing therapies that treat, both human and canine diseases. We therefore set out to verify an irradiation method for recipient testis preparation and to perform SSCT in the dog, as needed steps toward transgenesis in this model system. We demonstrate for the first time evidence of successful SSCT in the dog, helping achieve these goals.

Materials and Methods

Experimental design

The first part of this study involved confirmatory testing of a protocol to deplete endogenous male germ cells in dogs through the use of focal irradiation. Prepubertal dogs had their testes irradiated and then the testicular architecture and extent of recovery of spermatogenesis were tested 8 weeks after treatment. In the second phase of the study, mixed germ cells were isolated from donor testes and were transplanted into prepubertal recipient dogs that had had their germ cells depleted using that irradiation protocol. Sperm were collected from the recipients and genotyped to determine the origin and the relative contributions from endogenous versus donor spermatogenesis. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University.

Preparation of recipients

Two 5 month-old hound mongrels (Marshall BioResources, North Rose, NY) had their testes subjected to focal external beam radiation to deplete their endogenous germ cells as described (Kim, Selvaraj *et al.* 2006), with modifications for the larger size of dog testes. Briefly, the dogs were anesthetized and irradiated locally on the testes with a 6 MV linear accelerator (10 MeV electrons, dose rate of 300 MU/minute). A 5 cm-diameter cone was used to collimate the electron beam to irradiate both testes while minimizing exposure of surrounding normal tissue. A fractionated dose of 3 Gy was applied to the testes daily for three consecutive days, for a total dose of 9 Gy per animal. At 8 weeks after treatment, the testes from these dogs were removed for the evaluation of spermatogenesis for the first study. After confirming the efficacy of this protocol at depleting male germ cells while leaving testicular architecture intact, five other dogs were subjected to focal external beam irradiation of their testes by this same method to prepare them to be recipients for the SSCT procedure.

Germ cell isolation and culture

Mixed germ cells were isolated from donor testes by sequential enzymatic digestions, first using collagenase then trypsin (Kim, Selvaraj *et al.* 2006). After washing, the cell pellet was resuspended in Dulbecco's Modified Eagle's Media (DMEM) containing 10 % FBS, 100 mg/ml streptomycin sulfate and 100 IU/ml penicillin at a concentration of 67 - 100 million cells per ml. Two weeks prior to the transplantation, we isolated mixed germ cells from three sets of donor testes. These were maintained in culture. The culture medium contained 2 mM L-glutamine, MEM non-essential amino acids, and 20 ng/ml of

human Glial Cell Line-Derived Neurotrophic Factor (GDNF, R&D systems Inc., Minneapolis, MN) in addition to the components of the collection medium noted above. The cultured cells were harvested with 0.25 % Trypsin-EDTA and resuspended in the culture media at a concentration of 1.2 million cells per ml. Fresh and cultured cell suspensions were kept on ice until the transplantation.

Transplantation of mixed tubule cells including SSC into testes

At 8 weeks after irradiation, five prepubertal dogs were used as recipients for the transplantation. The animals were sedated using acepromazine (0.02 mg/kg) and butorphanol (0.22 mg/kg), and anesthesia was induced with propofol (4 mg/kg) and maintained with isoflurane (1 – 2 %). The dogs were placed in right lateral recumbency and the scrotal region was prepared for an aseptic procedure. The mixed germ cells were injected into the rete testis under the guidance of ultrasound scanning as previously described (Honaramooz, Behboodi *et al.* 2003), with minor modifications. An intravenous catheter (22G x 1 inch) was inserted through the caudal pole of the testis into the rete testis. Approximately 1 ml of cell suspension was injected into each testis until resistance was felt. The spread of the cell suspensions in the testes was monitored by ultrasonography from both sagittal and transverse planes.

Evaluation of sperm production from the recipient testes

Beginning 7 months after SSCT, manual semen collection was attempted from the recipients. However, the animals did not respond (unlike intact males from the same facility collected by the same individual during the time of this study, data not shown), over a period of 5 months. Blood was

collected from the recipients to analyze serum testosterone level by radioimmunoassay at Cornell's Animal Health Diagnostic Center (Ithaca, NY). After repeated failed attempts at manual collection, at 12 months post transplantation the recipients were castrated and their testes were collected and transported immediately to the laboratory. Epididymal sperm were harvested in Tris buffer (0.25 M Tris, 8.8 mM citric acid, 7 mM fructose in 100 ml of distilled water, pH 6.5) at 37 °C and the motility and number of sperm were examined. DNA was extracted from the sperm for genotyping analysis and the testes were fixed in Bouins' solution and processed for histologic examination.

Genotyping of the recipient sperm

The sperm were lysed in sperm lysis buffer (20 mM Tris-HCl, pH 8.0, 20 mM EDTA, 200 mM NaCl containing 80 mM DTT, 4 % SDS and 2 mg/ml proteinase-K; adapted from a user-developed protocol, QIAamp[®] DNA mini kit; www1.qiagen.com/literature/protocols/pdf/AQ03.pdf, Qiagen Inc., Valencia, CA) at 55 °C for a minimum of 7 hours. Then DNA was extracted with phenol/chloroform. Microsatellite analysis was performed to detect the presence of sperm derived from the donor in the recipient semen. For this, comparisons were made between donor and recipient genomic DNA versus DNA extracted from the sperm. Amplification reactions were performed in volumes of 25 µl containing 50 ng of template DNA, reaction buffer (10mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂), 200 µM dNTP (Fisher Scientific, Pittsburgh, PA), and 0.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA) in a Mastercycler gradient PCR machine (Eppendorf, Westbury, NY). The protocol was as follows: initial denaturation for 3 min at 94 °C; 94 °C for 15 s,

55 °C for 15 s, 72 °C for 30 s for 10 cycles and then 89 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s for 20 cycles followed by a final extension cycle of 5 min at 72 °C. One microliter of each PCR product was mixed with 0.2 µl of Genescan 500 LIZ size standard (Applied Biosystems, Foster City, CA) and 18.8 µl of formaldehyde for DNA fragment analysis using an Applied BioSystems 3730xl DNA Analyzer. The results were analyzed by GeneMapper Software v3.0 (Applied Biosystems).

An absolute quantitative PCR was performed using an ABI 7500 Fast Real-Time PCR system to establish the percentage of donor-derived sperm in comparison to a set of standard curves. TaqMan probes carried fluorescent 6-FAM and VIC as reporter labels at the 5' end for donor and recipient alleles, respectively, and a 'minor groove binder and non-fluorescence quencher (MGB/NFQ)' as a quencher at the 3' end. To verify the quantitative accuracy of this approach, donor DNA was mixed at specific ratios with that of the recipient in preliminary tests (data not shown). This led to the generation of two types of standard curve. The first curve was derived for absolute amounts of donor and recipient genomic DNA. This served as a baseline against which serial dilutions of PCR products from each marker were compared. In addition, specific mixtures were created with different ratios of recipient and donor genomic DNA (90:10, 85:15, 80:20, and 75:25; recipient:donor). The final amplification reaction (10 µl) contained 10 ng of sperm DNA template, 500 nM of forward and reverse primers, 150 nM of donor and recipient probes and 1 x TaqMan® Fast Universal PCR Master Mix (Applied Biosystems). A two-step PCR was used, 3 s at 95 °C and 30 s at 60 °C for 40 cycles following one cycle of 20 s at 95 °C. The microsatellite markers and the primers and probe for qPCR are listed in Tables 5.1 and 5.2, respectively.

Table 5.1. Microsatellite markers

Chromosome	Markers	Type	Forward primer	Reverse primer
CFA 1/6	FH2294	tetra	5'-GGGTCCCTGGGATCAAGCC	5'-TTACTAAAGAGACAGCTGATTGCC
CFA 11/7	FH2319	tetra	5'-GGAACACTGTATCCTCAGTGTC	5'-TGGGAAGGAAGGAAAGTGTG
CFA 15/6	FH2360	tetra	5'-CATTGATGCTGAATTTGACTTC	5'-TTACACACACTATTTTGTGGTCA
CFA 33/4	FH2165	tetra	5'-GGATGGAGTCCCACATCG	5'-CCTCACCCCAAATTCCTTAGC
CFA 34/6	FH2377	tetra	5'-TCCCTTGGGAAGTAGAGTG	5'-TAGCTAATGTGGTTAACGGTTACC
CFA 37/5	FH2532	tetra	5'-CACGCAGAAAGGCAGAAAG	5'-TTTCCATAGTGGCTGCATCA

Table 5.2. SNP markers and probes for qPCR

Chromosome	Markers	Forward primer	Reverse primer	Donor probe	Recipient probe
chr4:3180479	BICFG630 J72309	5'- tggggtcigacaggtttctc	5'- gcgggcctttaaaatatga	5' 6FAM- tctcatgcacGctccaa	5' VIC- tctcatgcacActccaa
chr5:3209630	BICF233J 57298	5'- acatggccacttgtttcag	5'- accctcggcccacttctat	5' 6FAM- agaacttttGcaaagcaga	5' VIC- agaacttttAcaaagcag

Statistical analysis

Linear regression was performed by JMP 7.0 (SAS Institute Inc., Cary, NC) and ANOVA was performed by Kaleida Graph 4.0 (Synergy Software, Reading, PA). For statistical hypothesis testing, the P value was set to $\alpha = 0.05$.

Results

Effect of irradiation on male germ cell development

The effect on male germ cell development of the irradiation protocol of 3 Gy/day for 3 consecutive days was first tested in the cat (Kim, Selvaraj *et al.* 2006), as was the proper timing for transplantation post irradiation. However, because different species exhibit different radiosensitivities, we investigated the efficacy of this protocol at depleting male germ cells in the dog. We irradiated 2 dogs as an initial test and castrated them at 8 weeks after treatment. The treated testes showed $\leq 5\%$ of seminiferous tubules contained spermatogonia at the 8 week time point, whereas full spermatogenesis was seen in all tubules in age-matched controls (Figure 5.1). There were no visible changes in the interstitial cell population or the gross testicular architecture between irradiated and untreated testes.

Evaluation of recipient testes and sperm

We next irradiated the testes of 5 more dogs to prepare them as recipients for SSCT. At 8 weeks post irradiation, transplantation was performed into 3 dogs with mixed tubule cells that had been maintained in culture for 2 weeks in an effort to expand SSC numbers. As the dogs were

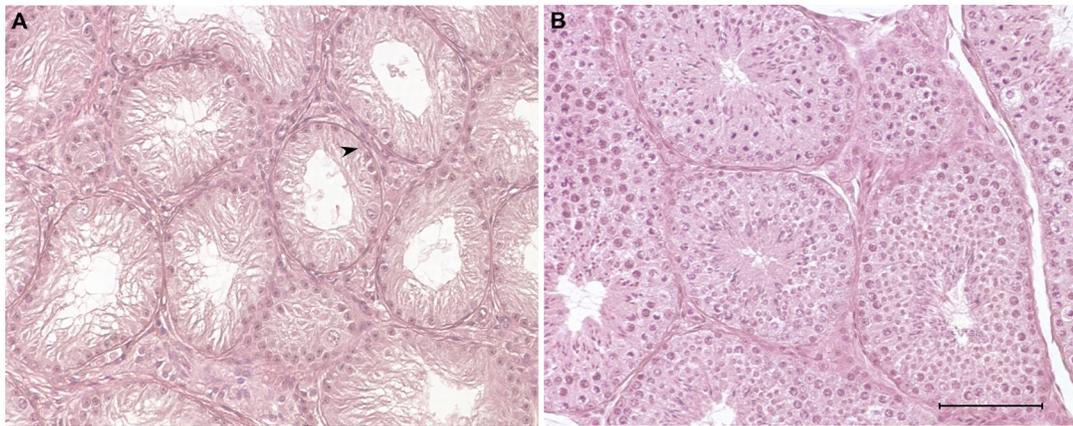


Figure 5.1. Cross section of seminiferous tubules from irradiated and untreated dog testes. Histological appearance of a treated testis 8 weeks after irradiation (A) and an age-matched, untreated control testis (B). Spermatogonia were seen as the only spermatogenic cells in the treated testes (arrowhead in panel A), whereas full spermatogenesis was observed in the seminiferous tubules of a 7-month old control animal. Bar = 100 μm .

recovering from anesthesia, they began to show an allergic reaction with generalized urticaria and pruritus. The symptoms resolved quickly after I.V. injections of diphenhydramine (40 mg/kg) and dexamethasone (2 mg/kg). Throughout the study, the testes of these 3 dogs remained uniformly small, and repeated attempts at manual semen collection failed between 5-12 months post transplantation. Therefore, we castrated them at this time. Histological sections revealed that >95% of tubules had a Sertoli cell-only phenotype (Figure 5.2), with the remaining tubules having some spermatogenesis that proved to be completely of recipient origin. Motility of epididymal sperm collected from these individuals was uniformly poor, visually assessed to be less than 5%.

The other 2 dogs were transplanted with fresh mixed tubule cells, in the base medium used for initial collection (i.e. without the glutamine, non-essential amino acids, and GDNF that had been added to our culture medium). These dogs had no allergic response, but also showed low libido and no interest in urine from a bitch in heat, and did not ejaculate upon repeated attempts at manual collection by a trained investigator. Therefore, these animals were also castrated at 12 months post-procedure for collection of sperm from the epididymides and histological inspection of the testes. The number of sperm collected from one recipient was higher than the other, but the percentage of motile sperm was similar between these individuals (R1: 1.8×10^7 sperm and 30% motile, R2: 8.2×10^5 sperm and 40 % motile).

Despite the lack of normal sexual behavior by any of the 5 dogs, serum testosterone concentrations for all 5 were within the normal range (1 – 7 ng/ml). Furthermore, there were no apparent changes in the histological appearance of Leydig cells or Sertoli cells.

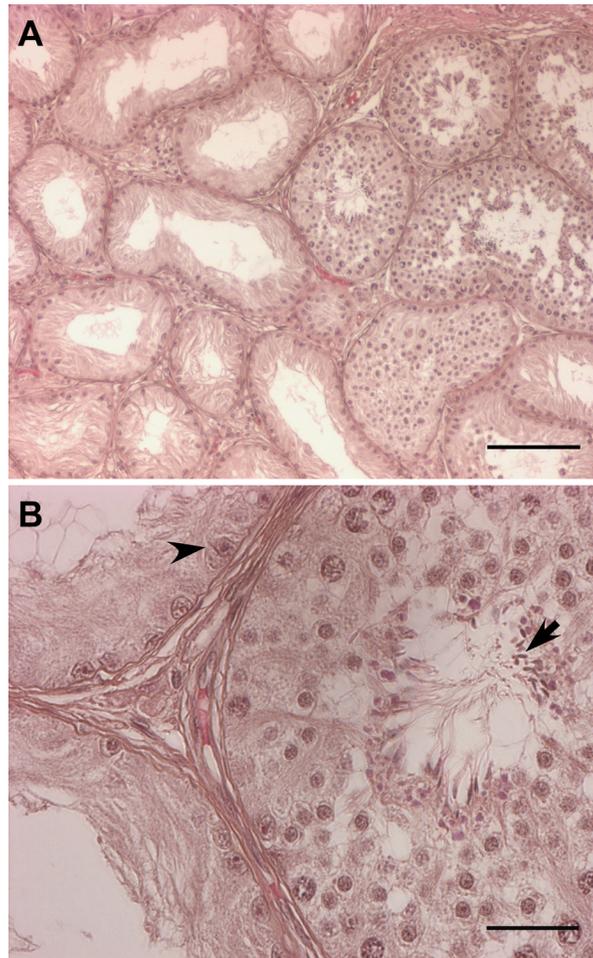


Figure 5.2. Cross-sections of seminiferous tubules after SSCT. Panel A shows both seminiferous tubules that have only Sertoli cells, and tubules that show full spermatogenesis (bar = 170 μm). It is impossible to discern whether this spermatogenesis is of donor or recipient origin by visual inspection in the absence of a protein marker specific for the donor (such as a transgene). Panel B is a close-up view of panel A (Bar = 30 μm). The arrow and the arrowhead in panel B indicate spermatozoa and Sertoli cell, respectively.

Genotyping of sperm collected from the recipients

Six microsatellite markers from the 'Microsatellite Multiplex Set-2' (Clark, Tsai *et al.* 2004) were identified as being informative to distinguish the donors (D1 and D2) and recipients (R1 and R2) (data not shown.).

Microsatellite analyses showed that R1 produced sperm derived from D1 SSC, by means of showing alleles of both the recipient and the donor (Figure 5.3). On the other hand, R2 showed no evidence of alleles representing donor D2 (data not shown).

Having identified donor-derived sperm in the epididymides of R1, we quantified the relative percentages of origin by performing real time PCR for SNPs that we identified as being able to distinguish between the donor and recipient (data not shown). Standard curves of serial dilutions yielded consistent and efficient amplifications. The parameters of the assays are shown in Table 5.3. Five replicates of the mixture-assay were performed along with the standard curve assay and there were no significant differences amongst the replicates (ANOVA, $p = 0.14$ and 0.26 , BICFG630J72309 and BICF233J57298, respectively). The ratio of donor alleles was calculated from each mixture based on the quantities of donor and recipient alleles that were obtained against the serial dilution-standard curves. The data set of each mixture produced linear regression lines ($r^2 = 0.93$ for both SNP markers) and the ratio of donor-derived sperm was predicted by the linear regression. The average percentage of donor-derived sperm was 19.5 % from BICFG630J72309 and 19.4 % from BICF233J57298 (range 19.0 – 20.1 % and 17.4 – 21.2 %, BICFG630J72309 and BICF233J57298, respectively). Both ranges of values were within a 95 % confidence interval (14.8 – 24.3 % and 15 – 23.8%, BICFG630J72309 and BICF233J57298, respectively).

Figure 5.3. Genotyping plots of microsatellite analysis. There are three plots for each marker, with the name of each marker indicated on the top of the first plot. The plots were obtained with recipient genomic DNA, DNA from sperm produced by the recipient 12 months after SSCT, and donor genomic DNA, in the top, middle and bottom rows, respectively. The Y-axis indicates the peak heights, the x-axis indicates DNA size, and the numbers in the boxes indicate the allele size of each peak. The microsatellite profiles for each marker show the presence of both recipient and donor alleles in the sperm DNA.

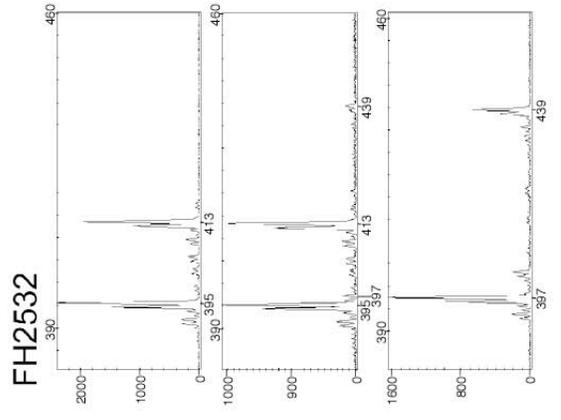
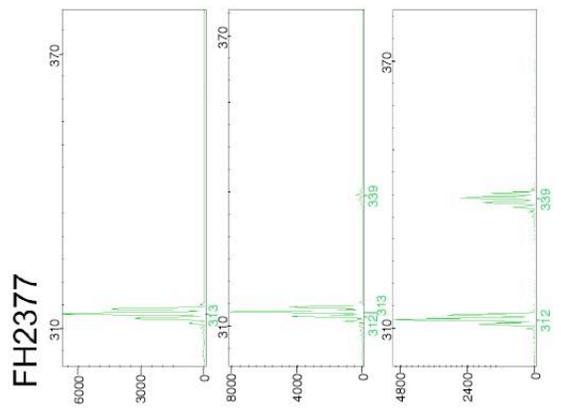
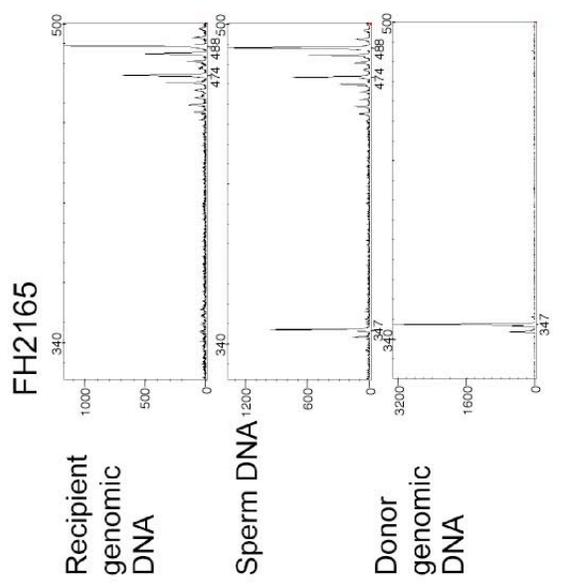
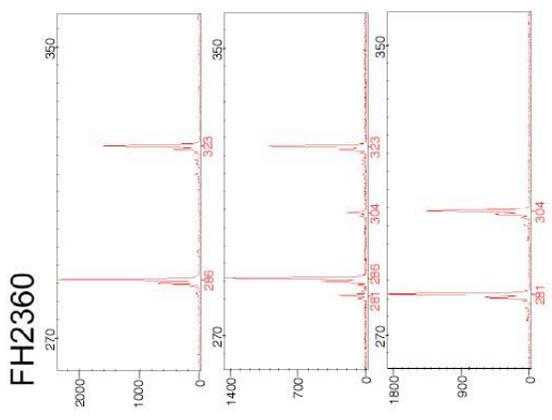
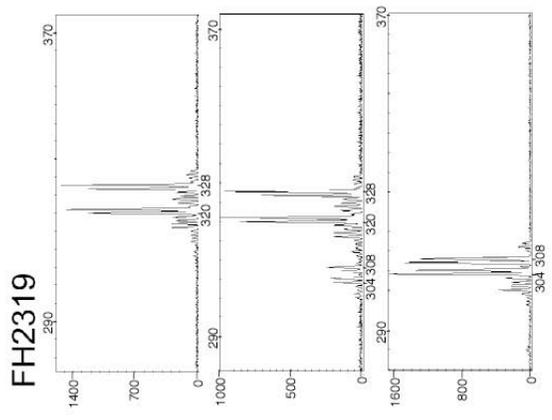
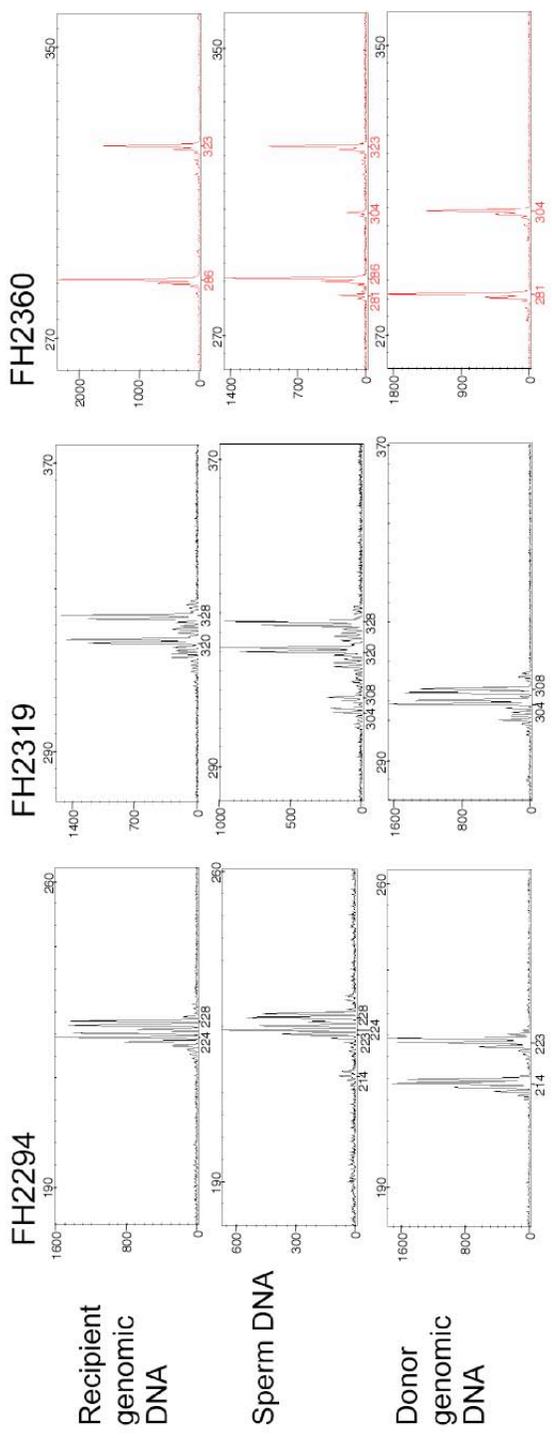


Table 5.3. Parameters of standard curves for both donor and recipient alleles of the SNP markers

	BICFG630J72309 D	BICFG630J72309 Rp	BICF233J57298 D	BICF233J57298 Rp
Slope ± SD	-3.65 ± 0.13	-3.98 ± 0.32	-3.70 ± 0.16	-3.90 ± 0.16
Y-intercept ± SD	40.64 ± 0.65	43.79 ± 1.99	43.77 ± 1.28	46.99 ± 1.68
r^2	0.999	0.995	0.998	0.995

D: probe of donor allele, Rp: probe of recipient allele, SD: standard deviation, r^2 : square of the correlation coefficient

Discussion

The dog is an important animal model to study human diseases because its genome and physiology are very similar to those of humans. Dog breeds have been developed over varying periods of time so that individuals display reproducible morphological, physiological and behavioral traits. This selective breeding has carried with it genetic predispositions to a variety of diseases that have counterparts to human diseases. Consequently, biomedical researchers can use these breeds to identify genes that are linked to certain diseases and traits with consequences for human and dog health (Khanna, Lindblad-Toh *et al.* 2006; Lindblad-Toh, Wade *et al.* 2005). The development of transgenesis would greatly enhance the full utilization of the dog as a genetic model, by allowing the effects of single gene alterations to be evaluated in a known genetic background. However, technical challenges imposed by canine reproduction have restricted conventional approaches to transgenesis. For example, the extremely dark cytoplasm of canine oocytes makes pronuclear injection difficult, and the high lipid content has thus far stymied attempts at oocyte or embryo cryopreservation. A few reports have been generated from one group about cloning dogs by somatic cell nuclear transfer (Jang, Hong *et al.* 2008; Jang, Kim *et al.* 2007; Lee, Kim *et al.* 2005). This would provide another mechanism to generate transgenic individuals; however, in many respects SSCT provides a technically more accessible alternative. A number of studies have shown that SSC can be transduced and used in SSCT to generate transgenic animals. This has been performed in mice, rats and goats (Honaramooz, Megee *et al.* 2007; Nagano, Brinster *et al.* 2001; Ryu, Orwig *et al.* 2006). Our study is the first to achieve SSCT in the dog, thereby providing an important step toward transgenesis in this species.

SSCT consists of three major parts: recipient preparation, injection of donor germ cells and analysis of sperm produced from the recipient. As the first part of the current study, we modified an irradiation protocol that we previously developed in cats (Kim, Selvaraj *et al.* 2006). In cats, we observed that about 10% of seminiferous tubules contained meiotic cells at 8 weeks post irradiation, whereas we observed that < 5 % of seminiferous tubules contained spermatogonia in recipient dogs at 8 weeks after the treatment. Because of good reduction in endogenous spermatogonia and no apparent histological changes in Sertoli cells or Leydig cells in the treated testes, we utilized this protocol for our transplantation experiments. However, abnormal sexual behaviors (e.g. no interest in urine from bitches in heat, no ejaculation upon repeated manual stimulations, and overall low libido) were observed from the 5 dogs that were irradiated and assessed for sperm production at 12 months post-transplantation. This failure could have been caused by either sub-lethal Leydig cell or Sertoli cell damage. It is also possible that too few endogenous germ cells remained to maintain normal communications with the somatic cell compartments, resulting in a reduction in overall testis function. The serum testosterone concentrations (6.35 ± 3.19 ng/ml, mean \pm standard deviation) were in the normal range of intact male dogs (1.0 – 7.0 ng/ml), arguing against a pronounced Leydig cell defect. Yet, it is possible that serum testosterone levels experienced spikes into the normal range but did not have normal variations. Continual monitoring of testosterone levels was not pursued, so the cause of the behavioral deficit remains unclear. Our findings suggest that the irradiation protocol should be modified in future attempts to promote overall testis function.

An alternative explanation might be that the recipient testes responded immunologically after SSCT. However, only the dogs receiving cultured cells responded with a visible allergic reaction, suggesting that a medium component (likely additional amino acids and/or GDNF) was responsible for the difference between the groups. No allergic or inflammatory response was seen in the 2 recipients of fresh mixed tubule cells, and the support of donor spermatogenesis in 1 of 2 recipients of the fresh cells strongly suggests that immune modulation of the recipient is not a strict requirement. In this regard, the dog would resemble other domestic animals such as pigs (Honaramooz, Megee *et al.* 2002), goats (Honaramooz, Behboodi *et al.* 2003; Honaramooz, Megee *et al.* 2007), and bulls (Herrid, Vignarajan *et al.* 2006).

Success in one individual in the current study showed that the transplanted SSC were able to support full spermatogenesis, resulting in epididymal sperm. In this individual, analyses of 6 microsatellite markers known to differ between the donors and recipients all showed mixed alleles. Quantitative PCR using TaqMan[®] probes based on SNPs estimated the percentage of the donor-derived sperm at 19.5%. Several methods have been used to estimate the amount of donor-derived sperm production from recipient testes after SSCT. These have included counting progeny (Brinster and Avarbock 1994), or quantifying donor cells expressing a visible marker in the recipient testes (Dobrinski, Ogawa *et al.* 1999; Herrid, Vignarajan *et al.* 2006; Honaramooz, Behboodi *et al.* 2003; Honaramooz, Megee *et al.* 2002). Even though the approaches to quantification have differed, the percentage of donor-derived spermatogenesis has tended to fall within a range of 10-35%, which is similar to our result.

This study shows for the first time that SSCT can be performed successfully in the dog in the absence of any modulation of the recipient's immune system, and lays a foundation for the production of transgenic dogs using SSC that have been modified prior to transplantation.

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CHAPTER 6

An alternative approach to canine transgenesis
using canine embryonic stem cells

Abstract

Research animals have contributed greatly to biomedical research and human welfare. Among the species used as research models, the dog has been unique for the following reasons: the similarities of their genome and physiology to those of humans, and the unique genetic makeup in each dog breed that has resulted from artificial selection. Although breed predisposition to particular diseases makes finding the responsible genes an attractive pursuit, linkage analysis alone is not sufficient to understand roles of single genes outside the context of genetic background. Therefore, development of transgenesis will enhance the utility of dogs as a genetic model. In this study, we attempted to generate transgenic dogs using canine embryonic stem (cES) cells. To achieve this, we needed 1) to predict the timing of ovulation for the accurate collection and staging of blastocysts, 2) to derive cES cells, 3) to manipulate dog blastocysts, and 4) to transfer canine embryos. Because this last stage is essential for multiple approaches to transgenesis, and because this technique has only been performed successfully a handful of times in the world, we emphasized optimizing canine embryo transfer. Female beagles aged 1-5 years were either mated with males to serve as embryo donors or were used as recipients of embryo transfer. Embryos were collected at days 14-15 post LH surge and 87.9% of the collected embryos were at blastocyst stage. Fresh embryos were transferred to recipient bitches at day 14-15 and 41.6% of blastocysts implanted and all were born as healthy puppies. To derive cES cells, the inner cell mass (ICM) was isolated from blastocysts. Although some of these cells developed into ES cell-like colonies, none of them was able to be established as ES cell lines. We succeeded in developing a methodology to penetrate canine blastocysts. We used this approach to

inject putative cES cells from another laboratory and then transferred these chimeric embryos into recipients. Although we observed 7 implantation sites (out of 8 embryos transferred), all embryos died and were resorbed. Data obtained some months later suggested that the ES cell line we used was subject to chromosome instability, suggesting a possible cause of embryonic mortality. Therefore, in the current study, we did not generate a transgenic dog from the cES cell-injected blastocysts. However, we demonstrated that canine transgenesis mediated via ES cells is technically feasible based on the high success rate of embryo transfer and our ability to manipulate blastocysts.

Introduction

Since the first successful transgenic mouse was made by microinjection of foreign DNA into embryos in 1980 (Gordon, Scangos *et al.* 1980), the vast majority of transgenic animals have been mice. The utility of these animals as disease and research models has been tremendous. Over time, transgenesis has been applied to other species such as rats, rabbits, goats, pigs and cows, and these animals are being widely used in the areas of agriculture, medicine and industry (Margawati 2003). Transgenesis in farm animals could quickly lead to the establishment of breeds with a particular trait. For example, transgenic cows might produce more milk, or milk with less lactose or cholesterol, pigs and cattle could produce more lean meat, and transgenic sheep could grow more wool. For medical applications, pigs might provide organs for xenotransplantation of human hearts, livers or kidneys. In addition, milk-producing farm animals have been generated that produce particular human proteins such as insulin, growth hormone and anti-coagulant factors in their milk (Maga, Shoemaker *et al.* 2006; Niemann and Kues 2003). Although

these animals play a critical role in biomedical research and human medicine, they have limitations in serving as models of human disease. Because there are significant differences in the genetic and physiological profiles of humans and mice, the development of appropriate mouse models for a large number of critical human diseases has been difficult. Agricultural species such as sheep and cows also have dramatically different physiologies. Therefore, the development of other mammalian models with a more similar genome and physiology to humans is highly desirable. In this regard, the dog has been an important animal model to study human diseases because their genome and physiology is much more similar to humans. Moreover, recently completed sequencing of the dog genome is facilitating investigations of diseases occurring in both humans and dogs. Over 360 naturally-occurring canine genetic diseases have been shown to have counterparts in humans, including forms of cancer, blindness, deafness and congenital heart disease (Sutter and Ostrander 2004). Moreover, several diseases have been shown to involve the same gene in both species, such as muscular dystrophy and bleeding disorders (Ostrander and Wayne 2005).

More than 400 different dog breeds have been established by artificial selection, which has resulted in not only unique morphological and behavioral characteristics but also susceptibility to certain disease for each breed (Sutter and Ostrander 2004). This predisposition allows identification of the gene(s) responsible for a particular disease. Although, linkage analysis has verified a number of genes related to specific diseases (Ostrander and Wayne 2005; Tsai, Clark *et al.* 2007), this analysis will not be able to fully explain the role of that single gene in a pathological condition. Therefore, transgenic dogs should be available for the full utilization of this model. However, limitations in our

understanding of dog reproduction and our ability to manipulate canine germ cells and embryos have prevented the full utilization of the dog as a genomic model. DNA microinjection, retrovirus- and embryonic stem (ES) cell-mediated gene transfer are conventional methods to generate transgenic animals. However, application of these methods to generate transgenic dogs has been hampered by difficulty in microinjection due to the highly opaque canine zygote, the current inability to manipulate canine early embryos, and the previous unavailability of canine ES cells. Therefore, we attempted to derive ES cells as a means to produce transgenic dogs.

ES cells are derived from inner cell mass (ICM) of blastocysts. They are pluripotent and can maintain an undifferentiated status *in vitro* in defined culture conditions. The first well-characterized ES cells were derived from mouse preimplantation embryos (Evans and Kaufman 1981; Martin 1981). About two decades later, primate ES cells (Thomson, Kalishman *et al.* 1995) were derived as well as human cell lines (Thomson, Itskovitz-Eldor *et al.* 1998). Because ES cells can differentiate into multiple cell types under different conditions, they can potentially be an important resource for treating diseases (Kiessling and Anderson 2006). The following traits are thought to characterize ES cells: mRNA and protein expression of stem cell markers such as Oct3/4, stage-specific embryonic antigen (SSEA)-3, SSEA-4, Tumor rejection antigens (TRA), and Nanog; alkaline phosphatase activity; ability to differentiate into cells from all three germ layers (endoderm, mesoderm, and ectoderm); differentiation into a specific cell lineage; generation of teratoma and chimeric animals (Conley, Young *et al.* 2004; Eiges, Schuldiner *et al.* 2001; Longo, Bygrave *et al.* 1997; Thomson, Itskovitz-Eldor *et al.* 1998; Thomson, Kalishman *et al.* 1995). The ability of ES cells to integrate into the

ICM of blastocysts, thereby generating chimeras, facilitates transgenesis using these cells if their genomes have been manipulated (Gossler, Doetschman *et al.* 1986). A number of transgenic mice have been generated by this approach including knockout mice, which are the most popular animal models for studying the functions of specific genes. Even though there have been a few reports about canine embryonic stem cell-like cells (Hayes, Fagerlie *et al.* 2008; Shingo Hatoya, Ryuzo Torii *et al.* 2006), the reported cell lines have not shown the complete set of required characteristics. Notably, none of these cell lines have contributed to the germ line. Very recently, after the current studies were completed and during the writing of this dissertation, a report has been published that describes a cES cell line with the ability to form teratomas (Vaags, Rosic-Kablar *et al.* 2008). The existence of this cell line makes the studies described below of even higher relevance, as our development of these technologies might enable that cell line to be used to generate transgenic dogs.

ES cell-mediated transgenesis also requires a set of skills including blastocyst manipulation (for the injection of ES cells into the embryo), and embryo transfer. Although embryo transfer is a common technique for some species including laboratory rodents and agricultural animals, there are only a few reports of successful canine embryo transfer leading to production of normal offspring (Lee, Kim *et al.* 2005; Tsutsui, Hori *et al.* 2006; Tsutsui, Hori *et al.* 2001b; Tsutsui, Hori *et al.* 2001c), primarily due to a lack of understanding of the reproductive physiology of female dogs. For example, canids are unique in ovulating a primary oocyte.

Previous studies demonstrated that early embryos, at the 2-cell to blastocyst stage, are suitable for transfer (Hori, Hagiuda *et al.* 2005; Tsutsui,

Hori *et al.* 2006; Tsutsui, Hori *et al.* 2001a; Tsutsui, Hori *et al.* 2001c).

Recently, dogs cloned by the somatic-cell nuclear transfer method have been reported (Jang, Hong *et al.* 2008; Jang, Kim *et al.* 2007; Lee, Kim *et al.* 2005), and the initial report claimed that the transfer of very early-stage embryos (4 hr after oocyte activation) is crucial in successful embryo transfer for dogs (Lee, Kim *et al.* 2005). However, in our study, we focused on blastocyst-stage cells for embryo transfer in the dog because the blastocyst would be the optimal stage to induce transgenesis by injection of cES.

Canine estrus occurs in a highly random pattern with the inter-estrous interval varying in bitches from 16 to 56 weeks (Bouchard, Youngquist *et al.* 1991; Christie and Bell 1971). This has significantly hindered canine reproductive research because of the costs associated with maintaining the very large numbers of bitches needed. In this regard, artificial estrus induction would be beneficial to generate synchronized donor and recipient bitches. A number of hormonal methods to induce estrus in bitches have been reported, including exogenous gonadotropins (Cain, Cain *et al.* 1988; Kusuma and Tainturier 1993; Shille, Thatcher *et al.* 1984; Vanderlip, Wing *et al.* 1987), dopamine agonists (Beijerink, Dieleman *et al.* 2003; Rota, Mollo *et al.* 2003; Spattini, Borghi *et al.* 2007; Zoldag, Fekete *et al.* 2001), and GnRH analogs (Cain, Cain *et al.* 1988; Inaba, Tani *et al.* 1998; Vanderlip, Wing *et al.* 1987; Volkmann, Kutzler *et al.* 2006a). An alternative, indirect method has also been used. In this approach, exogenous androgens (e.g. mibolerone) are applied in late anestrus, and then are withdrawn. This protocol is designed to release inhibition and allow a treated cohort to come into estrus with greater synchrony than if left untreated. However, the efficacy of these methods is highly variable. To avoid the need for synchronization, frozen embryos can be used

for more efficient embryo transfer. However, successful cryopreservation of canine embryos has yet to be reported, largely because of the high lipid content in canine oocytes/embryos.

In this study we investigated the derivation of canine ES cells, the synchronization of estrus, the cryopreservation and transfer of embryos, and the manipulation of canine blastocysts.

Materials and Methods

Reagents were purchased from Invitrogen, Inc. (Carlsbad, CA) unless otherwise stated.

Animals

Female dogs, aged 1-5 years, were purchased from Marshall BioResources (North Rose, NY). Three 5-year-old Labrador retriever sires were used for semen collection. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University.

Timing of ovulation and artificial insemination

Bitches were purchased in late anestrus as determined by their reproductive histories and serum progesterone levels, and were monitored every other day for signs of proestrus including vulvar swelling and seroganguineous vaginal discharge. Once one of these signs was observed, blood was collected for determination of serum progesterone (P4) levels by

ChemiLuminescent Immunoassay (CLIA, Cornell Animal Diagnostic Center, Ithaca, NY), every other day until P4 reached peripheral concentrations higher than 0.5 ng/ml. At that time it was collected every day until the concentration was over 5.0 ng/ml, at which point it was again collected every other day until passing 15 ng/ml. In addition, vaginal smears were obtained every other day until the first day of mating. Day 0 (the day of LH surge) was identified by a serum P4 level of between 1.5– 2.5 ng/ml. Artificial insemination was performed on day 2, 4 and 6 with fresh semen that was collected from one of the three breeding sires in our kennel. The number and motility of sperm were briefly examined using light microscopy to ensure semen quality.

Synchronization of estrus in bitches

To synchronize the estrous cycles of recipients and donors, mibolerone (100 mg/ml, RoadRunner Pharmacy, Phoenix, AZ) or Ovuplant® (2.1 mg of Deslorelin/implant) were used as shown in Table 6.1. Mibolerone was administered daily for 2–4 weeks according to the recommendations for Cheque-Drops®, the commercial mibolerone product that is currently unavailable in the United States. Ovuplant was inserted into the vulvar mucosa following local anesthesia, achieved first with with lidocaine HCL jelly, 2% (Alcorn, INC, Buffalo Grove, IL), followed by 0.2 ml of injectible Lidocaine. The implant was removed at 2–3 days post LH surge by making a small incision and scraping out the implant with sterile cotton swabs following local anesthesia as above. These methods follow the protocol of Kutzler (Kutzler 2005).

Collection of blastocysts

On day 14-15, the uterine horns and oviducts were removed from the donors via ovariectomy and then immediately transported to the laboratory in a warm container (~37 °C). The uterus was washed with warm sterile PBS (phosphate buffered saline, pH 7.4) and excess blood was blotted with sterile gauze. The embryos were flushed from each horn with 10 ml of flushing media containing DMEM (Dulbecco's Modified Eagle Medium), with 15% FBS (fetal bovine serum), and 100 IU/ml of penicillin and 100 µg/ml of streptomycin, through a 20 gauge needle inserted at the tip of uterine horns. The flushed embryos were recovered in complete TCM, which was TCM-199 supplemented with 15% FBS, penicillin and streptomycin, at 39 °C, in a 5 % CO₂ incubator until they were transferred or processed for ES cell derivation.

Feeder cell derivation

Primary canine embryonic fibroblasts (CEF) or mouse embryonic fibroblasts (MEF) were used as feeder cells. Dog and mouse embryos were collected at 30 and 14 gestation days, respectively, to derive the feeder cells. The derivation protocol was applied similarly to CEF and MEF. Briefly, the embryos were removed from embryonic sacs inside uterine horns under an aseptic hood. Visceral tissue was removed from the embryos and the remaining tissue was washed three times with 10 ml PBS. The cleaned embryos were minced into 3-4 mm-sized pieces, followed by addition of 2 ml of trypsin-EDTA solution and additional mincing. Another 5 ml of trypsin solution was added to the plate and the suspension was placed in a 37 °C incubator for 20-30 min. After the trypsin incubation, the mixture was pipetted vigorously up and down until having a relatively uniform, thick consistency, at which time DMEM supplemented with 10% FBS, penicillin and streptomycin was added.

Table 6.1. Embryo recovery and uses

Donor ID	Day of embryo recovery		no. CL	no. embryo recovered (developmental stage*)	use
	after LH surge	after mating			
4199863	14		3	2 (B)	
4477162	15		5	4 (B), 1 (H)	Fresh embryo transfer
4511140	-	10	6	2 (morulae), 4 (B)	
4352661	-	10	8	8 (B)	
4511573	14		7	2 (B), 4 (deg)	
4168224	14		5	1 (ExB), 3 (B), 1 (EB)	Frozen embryo transfer
4611225	14		5	2 (H), 3 (B)	
4260597	14		9	1 (H), 7 (ExB)	
4212916	14		9	6 (ExB), 2 (B)	
4295889	14		7	4 (ExB), 3 (B)	
4295731	14		8	2 (ExB), 2 (B), 2 (EB)	
4550099	14		7	3 (B), 3 (EB), 1 (deg)	
4791754	14		9	9 (B)	
5037867	14		8	6 (B), 1 (deg)	
4983424	14		8	4 (EB)	
4949552	14		6	6 (B)	

*: EB (Early blastocyst), B (Blastocyst), ExB (Expanded blastocyst), H (Hatched embryo), deg (degenerated)

5ml volumes were placed into a T75 flask containing 10 ml of the DMEM and incubated in a 37 °C tissue culture incubator overnight. The following day, the spent media was replaced and the cells were cultured until at least 90% confluent. The cells were harvested with a trypsin solution, and then were frozen with DMEM containing with 20% DMSO and 20% FBS and kept in LN₂. When needed, the cells were thawed and expanded over two passages prior to irradiation at 5500 rad for mitotic inactivation.

ES cell derivation

The irradiated feeder cells were plated at 0.75×10^5 cells/ml. The following day, blastocysts were collected from female dogs, the embryos were placed in the complete TCM on a 38.5 °C heated stage, and then the zona pellucida and trophoblast cells were removed by mechanical dissection. The isolated ICM were placed on either a CEF or MEF feeder layer in ESC medium, containing Dulbecco's modified Eagle's medium (DMEM)/F12 medium (DF12) containing 15% serum replacer, 0.1 mM nonessential amino acids, 1 mM L-Glutamine, and 4 ng/ml basic fibroblast growth factor (bFGF). The cells were monitored daily until the colony was deemed ready for first passage (usually 5-7 days), which was performed mechanically.

Manipulation and transfer of blastocysts

Putative canine ES cells, FhDo7, were obtained from Dr. Beverly Torok-Storb at the Fred Hutchinson Cancer Research Center (Seattle, WA) for microinjection into canine blastocysts. The cells were maintained in the same fashion as described in their manuscript (Hayes, Fagerlie *et al.* 2008). The FhDo7 cells expressed enhanced green fluorescence protein (eGFP) as a

result of transduction with a lentiviral vector containing the appropriate sequence. Therefore, we were able to use fluorescence to select ES cells from the mixture of the ES cells and mouse feeder cells after trypsinization with TrypLE. Three 7 μ l droplets of complete TCM for blastocysts, one 7 μ l droplet of cESC media for FhDo7 cells, one 3 μ l droplet of 0.25% pronase, and one 10 μ l droplet of complete TCM for rinsing tips were placed in the center of a 35 mm petri dish. The dish was flooded with mineral oil and maintained on a heated stage of an inverted microscope (Eclipse TE2000-U microscope, Nikon, Melville, NY) equipped with micromanipulators, and holding and microinjection pipettes (Eppendorf, Westbury, NY). Flushed blastocysts were placed in the complete TCM at 37 °C incubator with 5% CO₂ in air until microinjection and then each blastocyst was transferred to the 7 μ l-droplet of complete TCM. Green FhDo7 cells were selected with the transfer pipette from a mixture of MEF and the cells in the cESC droplet, and then moved to the droplet containing the blastocyst. The ES transfer pipette was filled with a small volume of 0.25% pronase taken from the pronase droplet. A hole was made in the zona pellucida by slowly releasing the pronase up against the wall of the zona pellucida until the tip could penetrate the hardened extracellular matrix. After removing the tip, it was placed in the rinsing droplet and washed thoroughly, prior to being used to pick up the FhDo7 cells. The tip was returned to the hole and the cES cells were injected into the blastocyst. The cES cell-injected and cESC media-injected (sham-control) blastocysts were kept in the complete TCM (+/- sodium butyrate depending on the experiment), and transferred into synchronized recipients immediately after the manipulations were finished.

For embryo transfer, bitches synchronized with the donors were used. They were anesthetized, clipped, scrubbed, and opened with a small ventral midline incision in the cranial abdomen. The uterus was visualized and inspected. A hole was made at the tip of the left uterine horn by a 16-gauge needle through which the manipulated fresh, or intact fresh or frozen embryos were transferred. A P200 Pipetteman with a large orifice, “cell-saver” tip was used to transfer the embryos in 70 μ l of the recovery media. A small suture was placed to mark the site of injection, and then the abdomen was closed using a standard 3-layer technique. Pregnancy was examined by ultrasonography on days 28–30 after the LH surge of the recipient. To time parturition, bitches were monitored for signs of distress, pain, inappetence, lactation, and changes in P4 and body temperature beginning on day 63. The pups were delivered by Cesarean section on day 65 after the LH surge.

Vitrifying and thawing blastocysts

Two methods were compared for vitrification of canine embryos. TCM 199 containing 10 % FBS, penicillin and streptomycin was used as a base medium for both. For treatment A, embryos were incubated in the base medium for 1 min and then moved to vitrification solution [7.5 % (v/v) ethylene glycol (Sigma-Aldrich, St. Louis, MO) and 7.5 % (v/v) DMSO (Sigma-Aldrich) in the base medium] for 3 min. The embryos were transferred to 17 % ethylene glycol and 17 % DMSO and 0.4 M Sucrose (Sigma-Aldrich) in the base medium and immediately loaded onto an EM-Grid (Ted Pella, Inc., Redding, CA). The embryos loaded on EM-Grids were plunged into LN₂ and kept in cryovials. For treatment B, embryos were pre-incubated in the base medium containing 7.5 μ g/ml cytochalasin B (Sigma-Aldrich) and 0.1 mM ascorbate

(Sigma-Aldrich) for 15 min and then processed as for treatment A. The embryos were kept in LN₂ until thawing before transfer.

To thaw the embryos, the EM-Grids were held in the air for 2 sec at RT and then immersed into 5 ml of 0.5 M sucrose in the base medium at 37 °C for 1 min. Cryoprotectant was removed by incubating the embryos in 5 ml of 0.25 M, 0.125 M, and 0 M sucrose in the base medium for 5 min successively. The embryos were immediately transferred into recipients.

Statistical analysis

ANOVA was performed by Kaleida Graph 4.0 (Synergy Software, Reading, PA). For statistical hypothesis testing, the P value was set to $\alpha = 0.05$.

Results

Synchronization of estrus

A total of 62 bitches were used for the studies in this chapter, which consisted of 4 different batches during a period of 3 years. Thirty-nine bitches were allowed to have natural onset of estrus and 10 of these were synchronized (25.6%). Eighteen bitches were treated with mibolerone resulting in synchronization of 6 bitches (33.3 %), whereas 5 bitches were treated with Ovuplant® and 4 of them were synchronized (80.0 %). However, 50% of the embryos collected from Ovuplant®-treated bitches were degenerated whereas no apparent defects were found in the embryos from mibolerone-treated bitches.

Embryo recovery

Table 6.1 shows the results of embryo recovery and their uses for embryo transfer experiments and Figure 6.1 shows the embryos collected on days 14-15. The time of ovulation (D0) was indicated by serum progesterone concentration except for two bitches, whose signs of cycling were not detected until after D0. On day 14–15, 90.0 % (99 embryos/110 corpus lutea) of the embryos were recovered from both uterine horns of 17 donor bitches and 87.9 % (87/99) of the collected embryos were at blastocyst stage. The embryos from mibolerone treated and untreated bitches didn't show any apparent differences regarding number or developmental stage ($p=0.46$). Some of them were at early blastocyst stage shown in Figure 6.1.A. but after 2-6 hours in culture, they expanded to regular blastocysts as shown in Figure 6.1.B.

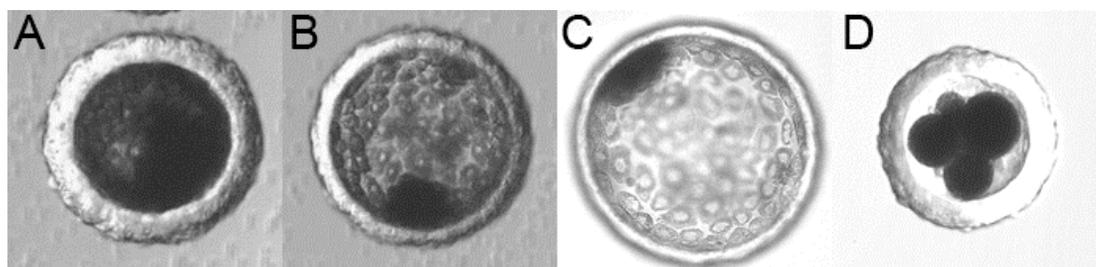


Figure 6.1. Canine embryos collected at 14 days post LH surge.
A. early blastocyst; B. blastocyst; C. expanding blastocyst and D. degenerated embryo.

ES cell derivation

Fifty-four blastocysts collected from 8 bitches were plated on either primary canine embryonic fibroblast (CEF) or mouse embryonic fibroblast (MEF) feeder layers. About 30% of ICM expanded as round, dark-brown cell clumps, with any attached trophoblast cells growing out in a flattened sheet atop the feeder cells (Figure 6.2). At 5-7 days of culture, the expanded group of cells from ICM was first passaged. After passage, some of the surviving ICM cells maintained the morphology of putative ES cell colonies whereas the rest of them began to differentiate at the outer edge of the colonies. Although a small number of colonies were maintained in culture over three passages, eventually all of them differentiated before they could be characterized for ES cell markers, and no stable canine ES cell lines were established.

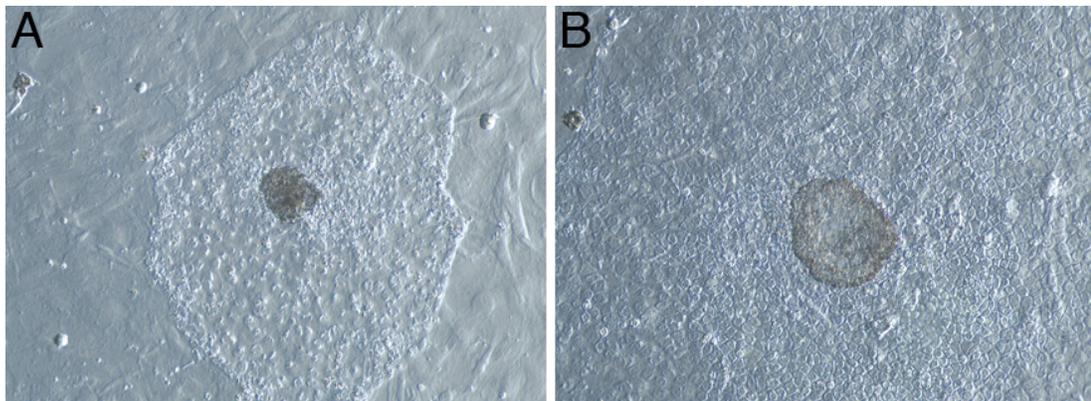


Figure 6.2. Expanded ICM on MEF in hESC culture media on day 4 (A, x100) and day 6 (B, x200). The dark brown colony in the middle of the images was derived from ICM and trophoblast cells are visible as a flat outgrowth around the group of ICM cells.

Embryo transfer

Table 6.2 shows the results of a series of embryo transfer experiments. When transferring fresh embryos, the day for the transfer was either day 14 or 15 for both donors and recipients. All four transfers on days 14-15 were successful in terms of observing embryo implantation and in terms of production of live offspring on day 65. The yield of 41.6 % (5 feti out of 12 transferred embryos) was identical to the number of implantation sites observed by ultrasonography, suggesting that there were no abortions or other developmental difficulties post-implantation. All puppies were born healthy and maintained normal health status until they were adopted. In the case of the first two offspring, these animals have maintained good health for a period of close to 3 years. Contact has not been maintained with the owners of the other 3 adopted embryo transfer puppies. All embryos used for vitrification were collected on day 14 from bitches that had estrus occur naturally. Frozen embryos all had normal appearance immediately after thawing;, however, none of frozen embryos transferred resulted in successful implantation.

In experiments in which blastocysts were injected with putative canine ES cells, again no live births were observed. On day 30 post LH surge, ultrasound was performed to detect pregnancy, but neither of the recipients had a live fetus. However, one of the recipients (ID#4773071) had 7 embryonic resorption sites in both uterine horns (4 on one side and 3 on the other side), whereas the other recipient did not have any evidence of implantation. The embryos that did not implant were transferred after incubation with sodium butyrate.

Table 6.2. Results of embryo transfer

	Donor ID	Recipient ID	Day of transfer after LH surge	no. embryo implanted	no. pup
Fresh ET	4199863 ^M	3328350 ^M	15	2	1
	4477162 ^M	3555429 ^N	14	4	1
	4511140 ^N , 4352661 ^N	4335490 ^N	-	4	2
	4511573 ^D	4513126 ^D	14	2	1
Frozen ET	4168224, 4791754	4454464	14	2 (A) 2 (B)	0
	4611225, 4550099	4652649	14	4 (A) 3 (B)	0
	4295731, 4550099, 4260597, 4791754, 4295889	4625153	14	10 (A) 5 (B)	0
	4212916	4331192	14	6 (A)	0
	4295889, 4295731	4460162	14	3 (A) 2 (B)	0
	5037867, 4983424	4773071	14	5 (E) 3 (S)	0
cESC injected ET	4949552*	5084121	14	4 (E) 2 (S)	0

M: Mibolerone treated, N: No treatment, D: Deslorelin treated, A: frozen in VSA, B: frozen in VSB, *: embryos treated with 0.2mM sodium butyrate prior to transfer, E: cESC injected, S: sham injected

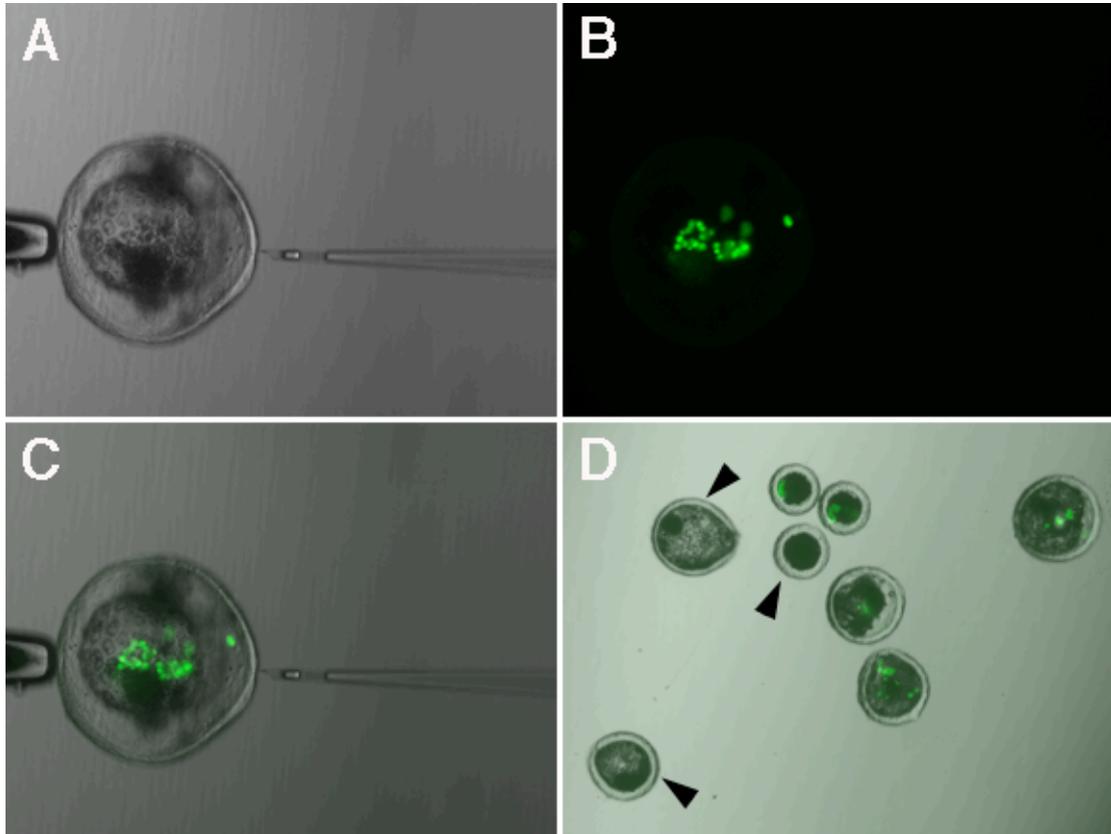


Figure 6.3. GFP expressed-cESC Injected blastocysts. Panels A, B, and C show the same blastocyst immediately after injection with green cESCs (bright field, green fluorescence and merged pictures, respectively). Panel D shows 8 embryos injected with either medium only (3 embryos, arrowheads) or green cES cells (5 blastocysts) prior to transfer.

Discussion

The development of biomedical research has been greatly advanced by the use of different species of research animals. Of these, the dog has been a unique research model for human medicine because its genome and physiology are similar to those of humans (Joy, Basak *et al.* 2006). Almost 400 different breeds of dogs have been established over a short period of time, yielding particular traits that are unique to each breed. As a result of this selection process, each breed has developed genetic predispositions to particular diseases and behavioral and morphological characteristics. Numerous laboratories are using the canine model to identify the genes linked to these diseases (Ostrander and Wayne 2005). However, linkage analysis cannot demonstrate how a gene is involved at the cellular level or is responsible for the disease pathologically. By providing a method to test the action of a single gene in a different genetic background, by providing the ability to mark specific cells and tissues, and by providing the ability to delete specific genes, transgenesis would allow the full utilization of this biomedical model.

There have been a small number of reports about derivation of cESC (Hatoya, Torii *et al.* 2006; Hayes, Fagerlie *et al.* 2008; Schneider, Adler *et al.* 2007; Vaags, Rosic-Kablar *et al.* 2008). Among the studies, Hayes *et al.* and Vaags *et al.* showed their cell lines have the most potential to be true ES cells, especially the cESC from the latter group, which were able to form teratomas. However, as of yet, there are no reports about chimeric dogs being generated with cESC. Our attempts to derive cESCs were part of our effort to develop transgenesis in the dog, because there were no reports of cESCs at the time. We tried both immuno- and mechanical dissection of blastocysts to isolate the

ICM and found that the immunodissection with anti-bovine trophoctoderm was not suitable for canine blastocysts because of the weak cross-activity of the antibody (data not shown). Mechanical dissection of blastocysts was preferred for isolating ICM as well as for subculture of primitive ES colonies, which is in agreement with the findings of other groups using cells from dogs (Hayes, Fagerlie *et al.* 2008; Vaags, Rosic-Kablar *et al.* 2008) as well as human cells (Thomson, Itskovitz-Eldor *et al.* 1998). The proliferative cell clusters derived from ICM initially maintained their ES cell-like appearance; however, they lost their undifferentiated status at an early passage, which prevented us from characterizing the cells. Although the derivation of cES cells was not successful in our laboratory, these attempts provided valuable information and experience with female reproductive physiology and early canine embryo development. Thus, these efforts laid the foundation for our successful canine blastocyst transfer, which itself we hope is a step toward canine transgenesis mediated by ES cells.

The reproductive physiology of a bitch is different from almost all other mammals. Oocytes are ovulated at the germinal vesicle stage and both stages of meiosis occur in the oviduct. Moreover, it has been shown that the onset of estrus is not accurately related to the time of ovulation. These unique characteristics have been obstacles in the development of assisted reproductive techniques in the dog. For example, more sophisticated oocyte culture conditions are required for *in vitro* maturation and *in vitro* fertilization. In addition, it is very difficult to time ovulation and synchronize multiple females to perform embryo transfer. However, we used a combination of vaginal cytology and serum P4 profiles to predict the retrieval date of blastocysts in the current study and these parameters were highly accurate (92.2 % of

healthy embryos was blastocysts). For our embryo transfer studies, the parturition date was calculated with the same parameters, as well as fetal measurements taken by transabdominal ultrasonography on D28-30. All of the puppies were born by Caesarian section on D65. Therefore, the measurements of P4 and vaginal cytology are reliable methods to predict the ovulation timing of a donor and to make sure that a recipient is ready with an appropriately prepared uterine environment. Additional monitoring with transabdominal ultrasonography and several observations of the pregnant bitches were together highly effective to choose an appropriate day for caesarian section.

In order to reduce the number of dogs needed, and to perform embryo transfer more effectively with less labor and expense, we investigated two different aspects of assisted reproduction. The first was estrous synchronization, and the second was embryo cryopreservation. For the first approach, we used either mibolerone or deslorelin in an attempt to induce synchronized estrus in the bitches. Our experimental design was based on previous reports of timing of estrus post-treatment (Kutzler 2005; Volkman, Kutzler *et al.* 2006b), and did not seek to determine the potential effects of these treatments on embryo quality. Mibolerone is an androgenic steroid approved for estrus suppression in the dog. According to the manufacturer, it can take 7-200 days for the bitch to resume the next cycle after mibolerone withdrawal. However, our co-investigator (Dr. Vicki M-W, James A. Baker Institute For Animal Health, Cornell University, Ithaca) used this drug in clinical practice, administering it daily to achieve at least a 6-month interval from the last estrus, to synchronize bitches at the late anestrus phase. After completing such a regimen, she observed that most bitches exhibited

proestrus approximately 2 weeks after mibolerone withdrawal. Similarly in this study, daily mibolerone treatment was begun in all bitches during anestrus and was maintained to synchronize them in late anestrus. All bitches in a housing group were then withdrawn on the same day. Estrous synchronization in each housing group was achieved through the "dormitory effect," wherein bitches in proestrus or estrus stimulate estrus induction in late anestrus bitches that are housed together (Concannon 1993). Another drug, deslorelin, has been studied for its efficacy in the synchronization of bitches (Kutzler 2005; Volkmann, Kutzler *et al.* 2006a; Volkmann, Kutzler *et al.* 2006b). Deslorelin is a GnRH analog, which stimulates the release of LH to grow follicles and induce ovulation. About 33 % and 80 % of dogs were synchronized among the dogs treated with mibolerone and deslorelin, respectively. However, about 50 % of embryos collected from the deslorelin-treated bitches were degenerated. These data suggested an unfavorable trade-off between synchronization success and embryo quality.

As an alternative to synchronization of bitches, cryopreservation of embryos can be used. This has never been reported in the dog. Therefore, we tested vitrification methods, which involve the immediate transition from a liquid into a glass-like phase, thereby inhibiting the formation of ice-crystals. This method has been widely used for gamete preservation (Beebe, Cameron *et al.* 2005; dela Pena, Takahashi *et al.* 2001; Menezo 2004; Wusteman, Robinson *et al.* 2004). However, the vitrification protocols used in this study did not preserve dog blastocysts successfully. All frozen-thawed blastocysts failed to implant or develop to fetus after transfer. While embryo transfer techniques were being established in our laboratory, putative cES cells were being derived in the laboratory of Dr. Beverly Torok-

Storb at the Fred Hutchinson Cancer Research Center (Hayes, Fagerlie *et al.* 2008). Although their cell lines showed various essential characteristics that define ESC—including differentiating into three different germ layers and expression of appropriate nucleic acid and antigenic markers—that group was not able to test the ability of their cells to contribute to the germline because of a lack of experience in canine reproductive physiology. As described above, the early canine embryo is surrounded by a zona pellucida that is in some way different from most other mammals, in that it is tougher and more elastic such that it cannot be penetrated by conventional methods including piezo drills or laser drills (Figure 6.4.B and data not shown). In our preliminary study, we observed that canine blastocysts can be penetrated by trickling pronase on a single point of the zona pellucida to create a small opening that can be used as a portal through which cells can be injected (Figure 6.4.C-E). These embryos maintained normal shape and appearance after the hole was made. This approach was needed because exposure to pronase in the droplet itself resulted in blastocyst death (data not shown). We applied this method to generate chimeric embryos in the current study. Although none of our cES- or sham-injected blastocysts developed past implantation, we observed 7 implantation sites out of 8 transferred embryos (3 sham and 5 cES injected) in the first trial. In that trial, inexperience resulted in a lengthy time to manipulate all 8 embryos, which resulted in a 9-hour interval between the flushing and the transfer. Considering that our successful transfers had a maximum 3-hour interval, we predicted that the long time spent in a non-ideal environment might have affected the fitness of embryos. Therefore, for the second trial, two major changes were applied: experience allowed us to manipulate the 6 embryos in 3 hours to eliminate the time problem, and the manipulated

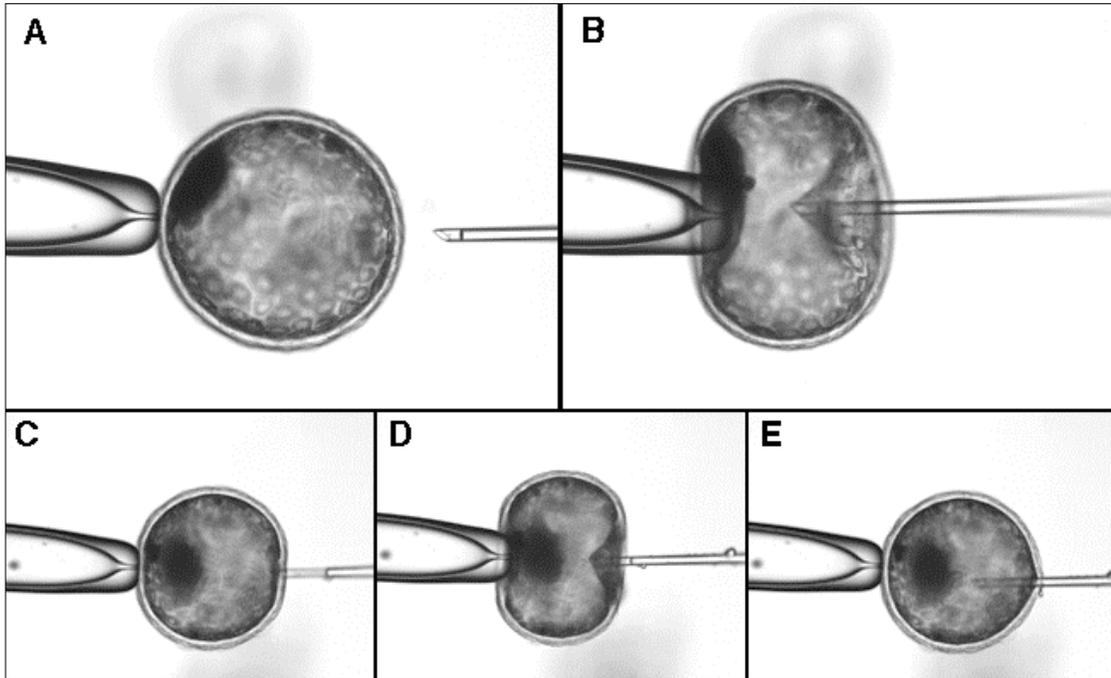


Figure 6.4. Canine blastocysts being penetrated for the injection of ES cells. Panel A shows a canine blastocyst collected on day 14, being held by a vacuum-operated holding tip on the left side of the panel. The zona pellucida surrounding the blastocyst was too elastic to be penetrated. A transfer tip can deform the embryo from one side to the other side where the holding tip is shown in panel B, and then the zona and the embryo retain their original shape. The same embryo was manipulated and penetrated by the transfer tip by gently applying 0.25% pronase on a single point of the zona pellucida. After washing the tip, the transfer tip could slowly be inserted into blastocoele as seen in panels C, D, and E. This blastocyst was approximately 400 μm in diameter.

embryos were placed in culture medium containing 0.2 mM sodium butyrate in an effort to improve the efficiency of transgenesis. This treatment resulted in better efficiency with mouse ES cells (personal communication, Dr. Carol Ware, University of Washington, Seattle, WA). However, there were no implantation sites found from this trial, suggesting either that time was not the major problem with this approach, or that sodium butyrate was not tolerated by the canine embryos, or that a different problem specific to this single trial was encountered.

In this study, we showed that canine blastocysts can be harvested and transferred with high yield, which differs with a previous report (Lee, Kim et al. 2005). In addition, we obtained favorable success rates with fresh embryo transfer in comparison with other reports of transfer at the morula-blastocyst stage. In addition, we obtained valuable comparative data on embryo quality using different synchronization regimens, and valuable negative data on canine blastocyst cryopreservation. Even though this project did not proceed further to result in chimeric offspring, we established significant technical progress toward the future generation of transgenic dogs.

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Chapter 7

Final discussions and Future directions

This study explored the potentials of spermatogonial stem cells for the preservation of male genetic information and for the development of transgenesis in the cat and dog, via the technologies of testis xenografting and spermatogonial stem cell transplantation (SSCT).

Lessons learned from studying testis xenografting

Testis xenografting was first reported in 2002, using testis tissue from mice, pigs and goats (Honaramooz, Snedaker *et al.* 2002). Since the first report, different species were used as donors including cattle (Oatley, Reeves *et al.* 2005; Rathi, Honaramooz *et al.* 2005), rabbits (Shinohara, Inoue *et al.* 2002), cats (Snedaker, Honaramooz *et al.* 2004), humans (Schlatt, Honaramooz *et al.* 2005), and horses (Rathi, Honaramooz *et al.* 2006). Although most of these studies showed that xenografts could produce sperm when the donor testis tissue was obtained from young animals such as neonatal mice, pigs, cows or primates, a small number of studies showed that adult testicular tissue degenerated in the host mice (Rathi, Honaramooz *et al.* 2006). An additional study compared multiple ages of donors for the success of bovine testis xenografting (Schmidt, de Avila *et al.* 2006), but the donors used were all neo-natal to pre-pubertal.

However, no studies delimited the precise window of donor ages that could be used for successful xenografting until our study using testes from domestic cats (Kim, Selvaraj *et al.* 2007). We found that the xenografts from pubertal cats failed to support post-meiotic germ cell development, despite producing normal amounts of bioactive testosterone. Our results also suggested a delay in sperm production, and some differences in the pattern of

meiosis in the tubule cross-sections. Given these differences versus the success of xenografting in many other species, I next compared the nature of spermatogenesis in the xenografts from different species in carnivore. Unlike the delay in sperm production by the cat xenografts, the progression of germ cell development in the ferret testis xenografts was very similar to that in the intact animals. However, canine spermatogenesis was poorly supported to produce sperm in the xenografts compared to cat and ferret xenografts. In the rare instance when spermatogenesis was supported (<1% of pre-pubertal grafts yielded sperm), it did not show the same delay as in the cat. Although our study did not identify the factor that caused the different response in these three species, we were able to conclude that the success of testis xenografting was affected by species-specific factors rather than by an effect for all carnivores.

Despite the new approach to preserve male genetic information that testis xenografting represents, there are several key points of research that must be investigated before the technique becomes widely used. Namely, what underlies the difference between species? What causes the precipitous decline in success at the time of puberty? And most important, are the sperm of different species that are produced by xenografts functional?

Lessons learned from studying spermatogonial stem cell transplantation

SSCT was introduced in 1994 using mice as donors and recipients (Brinster and Avarbock 1994). Although like testis xenografting, SSCT can be useful to preserve male genetic material from genetically valuable animals, this technology is limited in several ways. First, it is not simple to perform, requiring the isolation of germ cells from donor testes, the preparation of

recipients, and expertise in an effective method of retrograde injection. In addition, it requires a degree of taxonomic relatedness between the donor and recipient species for spermatogenesis to be supported. SSCT has been performed with various donor species including the goat (Honaramooz, Behboodi *et al.* 2003; Honaramooz, Megee *et al.* 2007), pig (Honaramooz, Megee *et al.* 2002), bull (Herrid, Vignarajan *et al.* 2006) or monkey (Schlatt, Foppiani *et al.* 2002). My studies expanded the number of species for which SSCT could be performed, including the development of an irradiation protocol to deplete endogenous male germ cells in the recipient cats (Kim, Selvaraj *et al.* 2006), and demonstration of successful SSCT in the dog, including donor cell isolation, recipient preparation, successful injection, and finally quantification of the relative yield of sperm of donor origin (Kim, Turner *et al.* 2008). Successful canine SSCT suggests that this approach might be useful in attempts to develop transgenesis in dogs.

Future directions

We demonstrated that feline testis xenografting could result in production of mature sperm. Because sperm produced in xenografts do not have outflow to the epididymis, they would eventually degenerate within the tubules of xenografts. We collected feline testis xenografts at different time points to determine the optimal time for harvesting sperm that would provide the best combination of viability and yield. Our results suggested that 45 weeks after xenografting was optimal. We then tested their abilities to fertilize eggs through intracytoplasmic sperm injection (ICSI) and support normal embryo development. We have not yet had success in generating normal blastocysts. For a next step, I propose that we test whether the sperm can

contribute to normal embryo development if they are co-injected with mature centrosomes collected from epididymal sperm. This approach has been shown to improve embryo production when feline testicular sperm were used for ICSI (Comizzoli, Wildt *et al.* 2006).

Although more than 70% of felid species and a high percentage of domestic cats show teratospermia (defined as ejaculation of <40% morphologically normal sperm), the cause(s) of this constellation of abnormalities remain unknown. Testis xenografting could be used to investigate teratospermia by comparing sperm production in a cat testis versus in the environment of a xenograft. If the problem were based within the germ cells, neither sample would produce normal sperm. However, if it was caused from an abnormal interaction with the neuroendocrine axis, sperm might be produced in the xenografts but not in the animal. This kind of approach could be complemented by genetic studies to determine whether there is an underlying genetic component to the abnormality. This study might provide insight into infertility in men, in addition to the cat. If a specific genetic defect were identified, one might then identify young males having the gene(s) and those with the normal gene profile, and perform SSCT to investigate the relative contributions of the germ cell versus the somatic cell compartments.

Unlike feline testis xenografts, canine spermatogenesis was rarely supported in xenograft. Therefore, the future study of dog testis xenografts should be focused on identifying the factor(s) causing the incompatibility between the canine tissue and the host murine system. As discussed earlier, canine FSH receptors should be the first candidate to study in this regard.

For SSCT in the rodent, the donor germ cells can be injected via the efferent ducts under a dissecting microscope (Brinster, Ryu *et al.* 2003;

Brinster and Zimmermann 1994; Parreira, Ogawa *et al.* 1998). Alternatively, ultrasonography has been used to guide the injection in the goats, pigs and bulls because the rete testis is hyperechoic, and the efferent ducts are buried within connective tissue between the tightly connected testis and epididymis, hindering direct injection in these species (Herrid, Vignarajan *et al.* 2006; Honaramooz, Behboodi *et al.* 2003; Honaramooz, Megee *et al.* 2002). However, neither of methods can be readily applied to cat testes because they are too tightly connected with the epididymis to utilize an efferent duct approach, and they are too small to apply an ultrasound probe and still manipulate the testis for injection into the rete. Therefore, I performed SSCT in the cat by injecting donor germ cells based on ratiometric landmarks without any visual guidance. However, the injection method was not appropriate to feed the cells into the rete testis and the seminiferous tubules, which resulted in our failure to obtain donor-derived sperm from all recipients. Although the morphology of cats has hindered the development of SSCT in this species, advancements in ultrasonography might overcome this obstacle [e.g. the Visualsonics Vevo 770 (http://www.visualsonics.com/products/products_vevo770.htm), Visualsonics, Toronto, Ontario, Canada]. Because this machine can be used on small animals such as mice, it should be able to be used on cat testes to enable visual guidance of injections into the rete.

In addition to the potential for SSCT to be used for the preservation of male genetic information, SSCT can also be useful to study spermatogonial stem cells and to generate transgenic animals. Transgenic mice (Nagano, Brinster *et al.* 2001), transgenic rats (Honaramooz, Megee *et al.* 2008; Ryu, Orwig *et al.* 2007), and transgenic goats (Honaramooz, Megee *et al.* 2008)

have all been generated using this technique. Because transgenic dogs have never been generated due to the unavailability of canine ES cells and the difficulty of manipulating early embryos, SSCT should be considered strongly as an alternative approach to develop transgenesis in the dog. Even though lentiviral vectors are the most common transgene-carriers for mitotically quiescent stem cells, the development of other types of transfection such as nucleofection should also be considered because viral transduction requires maintaining cells in culture, during which time they might lose their stem cell characteristics. Our attempts to generate transgenic dogs using canine ES cells and blastocysts failed due to the instability of the putative ES cells and the lack of financial support to continue the projects. As a future direction, I would focus on utilizing our zona penetration methodology as a means of injecting both transduced cells as well as directly injecting viral vectors into blastocysts. This approach should be attempted until the ES cell lines that are currently available can be demonstrated to be stable after repeated passages.

Unlike other adult stem cells (not including induced pluripotent stem cells), SSC have the unique ability of germline transmission (Kanatsu-Shinohara, Ogonuki *et al.* 2003), while also being able to transform into pluripotent stem cells under appropriate culture conditions (Kanatsu-Shinohara, Inoue *et al.* 2004). In the future, canine SSC should be characterized in terms of their gene and protein profiles, and surface antigens. These could be different from those seen in murine SSC and could be used to enrich the cells and verify their “stemness” after being maintained in culture. Culturing canine SSC has not been successful in either our laboratory or that of a collaborator (pers. comm. Dr. Beverly Torok-Storb), making empirical development of this methodology another potentially useful topic of research.

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