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Recipient Preparation and Mixed Germ Cell Isolation for Spermatogonial Stem Cell Transplantation in Domestic Cats

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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 3 consecutive days. These cats were castrated at 2, 4, 8, 16, and 32 weeks posttreatment, 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 were 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.

Key words: Testis, male, radiation, feline, conservation.
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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 postmortem 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 et al, 2003a; Kubota 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 millimeter-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 et al, 2002b), hamster and monkey (Schlatt et al, 2002b), calves (Oatley et al, 2005), rabbits (Shinohara et

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al, 2002), and cats (Snedaker 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 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 intracytoplasmic 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 et al, 1997) or via retrograde injection into the rete testis (large-animal models; Honaramooz et al, 2002a, 2003; Izadyar et al, 2003b). 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 on 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 estrous 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 donor and mice as recipients (Clouthier et al, 1996; Ogawa 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 et al, 1999; Nagano 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, 2 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 et al, 2003). Several techniques have been used to reduce or deplete endogenous male germ cells, such as irradiation (Withers et al, 1974; Meistrich et al, 1978; van Beek et al, 1990), chemotherapeutic drugs (Ogawa et al, 1997; Brinster et al, 2003), and cold ischemia treatment (Young 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 et al, 1990; van der Meer et al, 1992; Vergouwen et al, 1994). A number of radiation treatment protocols have therefore been tested and used to prepare recipients of several species for SSCT (Creemers et al, 2002; Schlatt et al, 2002a; Izadyar et al, 2003b).

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 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 (Bellvé et al, 1977b; Honaramooz et al, 2002a). 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, 2 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,

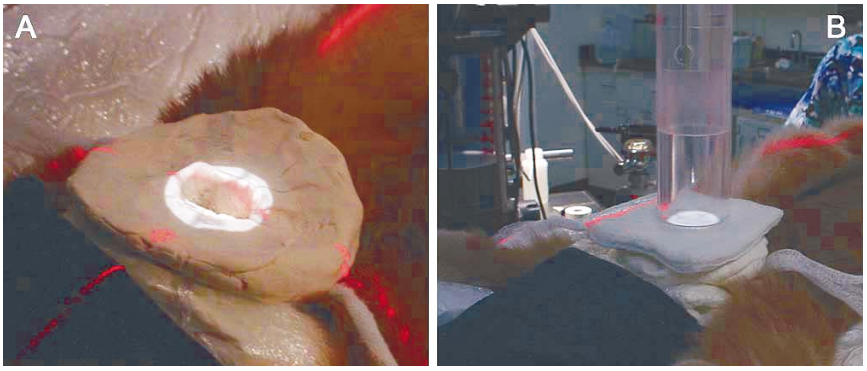

Figure 1. Positioning of the testes for external beam radiation treatment. The cat was positioned on a vacuum cushion repositioning device and the beam was centered precisely between the 2 testes **(Panel A)**. **Panel B** shows the collimating cone atop the tissue-equivalent material.

Fort Dodge, Ia), diazepam, and isoflurane (Abbott Laboratories, North Chicago, Ill) were used for the induction and maintenance of anesthesia in the experimental cats. For histology, 100% and 70% ethanol (Pharmco, Brookfield, Conn), hematoxylin, and eosin (Electron Microscopy Sciences, Fort Washington, Calif) were used. Trypan blue was purchased from Gibco (Carlsbad, Calif) 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 4 under standard lighting (12 hours light:12 hours dark) 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

Five-month-old domestic cats (n = 10) were anesthetized with ketamine HCl (5 mg/kg IV) and diazepam (0.5 mg/kg IV) and maintained by masking with isoflurane (1%–3%). Each cat was positioned in sternal recumbency using a vacuum cushion (Vac-Lok,™ Med-Tec, Orange City, Ia). The hind limbs were extended caudolaterally 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 1). The testes were irradiated with a 6-MV linear accelerator (7 MeV electrons, dose rate of 300 MU/min). 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 3 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 1 exposure.

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 Bouin 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 were observed under a light microscope at 100× 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, Mass). Statistical significance was assumed at $P < .05$.

Testis Histology and Evaluation

The fixed testes were washed out of the Bouin solution into 70% ethanol, then dehydrated in ethanol prior to embedding in paraffin and sectioning at 4 μm. 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 percentages 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 prepubertal, pubertal, and young-adult an-

Progression of spermatogenesis in treated and untreated control testes

Time After Treatment (wk)	Percentage of Tubule Cross Sections Containing:				Percentage of Tubule Cross Sections in Age-Matching 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 indicates spermatocytes; RS, round spermatids; and ES, elongating spermatids. Two treated animals and 1 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 nonsequential histological sections of each testis, and generating mean and standard deviation using Origin 7.0 software.

imals at a local veterinary hospital and animal shelters. A 2-step enzymatic digestion was performed, as described by Dobrinski et al (1999). Hanks 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-μm filter (Millipore, Billerica, Mass). After rinsing the testis in this medium, visible blood was blotted and the testis 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 μg/mL) as above. The resultant cell suspension was filtered through a 70-μm nylon mesh (BD Falcon, San Jose, Calif), washed by centrifugation at 600 × *g* for 5 minutes at room temperature three times, and resuspended in Dulbecco modified Eagle medium (DMEM) containing 100 μg/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 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 μL of cell suspension (10^8 cells/mL). To analyze the success of transplantation, recipient testes were collected between 48 and 456 days posttransplantation. The tunica albuginea was removed, and the seminiferous tubules were gently dispersed with collagenase prior to fixation in freshly prepared 4% paraformaldehyde for 2 hours 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 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 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, less than 1.5% of seminiferous tubules contained meiotic cells, compared with more than 50% of tubule cross sections in age-matched controls (the Table). In addition, most cross sections of tubules showed disarranged Sertoli cells (Figure 2A and C). At 8, 16, and 32 weeks posttreatment, 4%–9% of

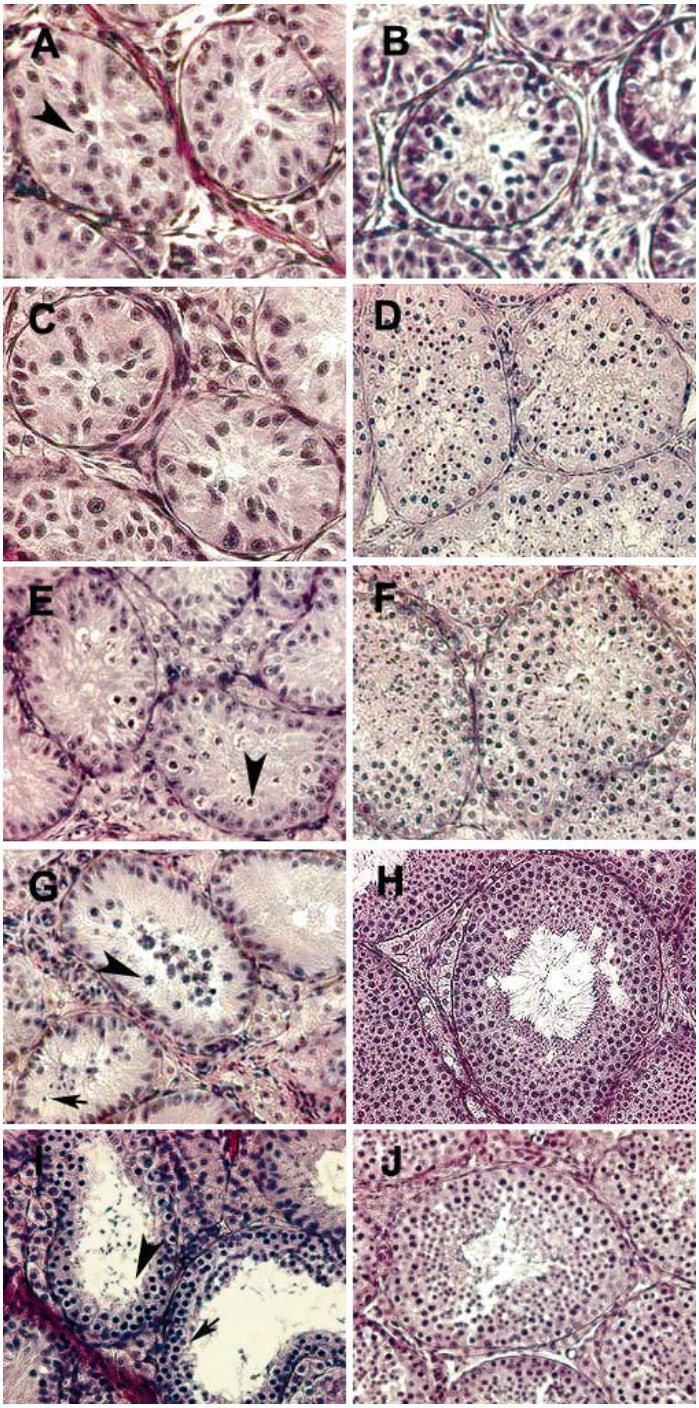

Figure 2. Histological appearance of irradiated and age-matched, untreated control testes. Irradiated testis tissue collected at 2 (**Panel A**; the arrow head points to a Sertoli cell), 4 **(Panel C)**, 8 (**Panel E**; the arrow head points to a spermatocyte), 16 (**Panel G**; the arrow head points 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 (200×).

seminiferous tubules contained spermatocytes, whereas full spermatogenesis was seen in all tubules in age-matched controls (Table; Figure 2). At 32 weeks after treatment, a very small number of tubules (<1%) had sperm, compared with age-matched controls (Figure 2I and J). There was no apparent change in the interstitial cell population between irradiated and untreated testes (Figure 2).

Isolation of Mixed Germ Cells

Successful mixed germ cell isolation in the cat relied on 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 prepubertal, pubertal, and adult cats. Approximately 90% of the cells excluded trypan blue, suggesting viability. Because of the large intercellular bridges connecting male germ cells (Ravindranath et al, 2003), spermatids often coalesced into larger, multinucleated cells (data not shown). This phenomenon is also seen during the separation of murine male germ cells (Bellvé et al, 1977a).

Assessment of the Presence and Viability of SSC Isolated During Germ Cell Dissociation

To demonstrate that viable SSC 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 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 3).

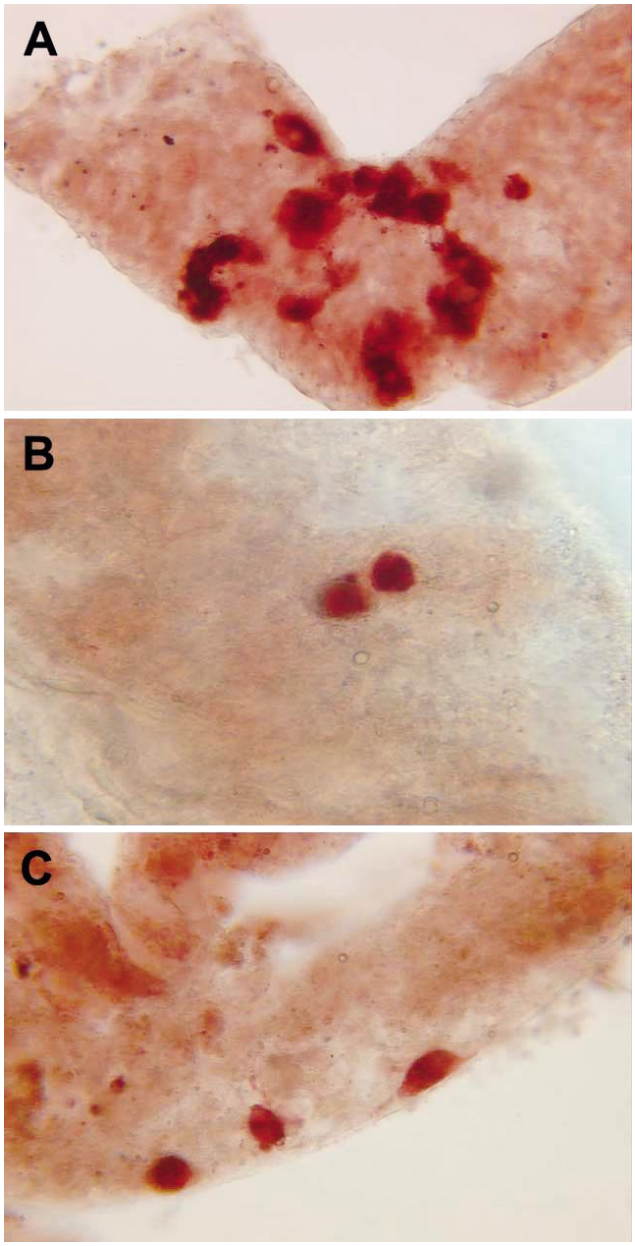

Figure 3. Detection of cat testis cells in mouse seminiferous tubules. Injected feline testicular germ cells colonized mouse seminiferous tubules and stained with an antibody specific for cat testicular cells in whole-mount immunohistochemistry on 63 days (**Panel A**, 16×), 120 days (**Panel B**, 40×), and 430 days (**Panel C**, 16×) 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 et al, 1996; Ogawa et al, 1997; Ogawa et al, 1999a), large domestic animals (Dobrinski et al, 2000; Honaramooz et al, 2002a, 2003; Izadyar et al, 2003b), dogs and rabbits (Dobrinski et al, 1999), and primates (Nagano 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 2 purposes: it increases the success of colonization by opening up the appropriate niches for the transplanted SSC (Spradling et al, 2001) and it improves the relative yield of donor-derived vs 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 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 et al, 1990; van der Meer et al, 1992; Vergouwen 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 et al, 2003b). When prepubertal rats were irradiated with a single dose of 3 Gy, all the research animals showed a resumption of endogenous spermatogenesis by 70 days posttreatment (Guitton et al, 2000). A 2-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 posttreatment, endogenous spermatogenesis was reduced to less than 10% of normal levels, suggesting that fractionated radiation protocols might improve duration of effect (Creemers 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 3 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 postprocedure so that sperm could be collected over the time period required.

Spermatogenesis in domestic cats usually begins when 5 or 6 months old (Tsutsui et al, 2004), and the spermatogenic cycle takes 46.8 days (França and Godinho, 2003). At 2 weeks posttreatment, 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 and 8 weeks, and spermatocytes began to be observed by week 8. The timing of this recovery suggests that approximately 4–8 weeks posttreatment 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 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 2 protocols, those of Bellvé et al (Bellvé et al, 1977b) and Dobrinski et al (Dobrinski et al,

1999), the latter of which proved more efficient for domestic cats. Before exposure to digestive enzymes, 2 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 multinucleated 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 SSC 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 on which to perform spermatogonial stem cell transplantation in the feline model system.

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