

Published in final edited form as:

Reproduction. 2008 December ; 136(6): 823–831. doi:10.1530/REP-08-0226.

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 spermatogenesis, but retention of normal testis architecture. Then, five month-old recipient dogs (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 injection 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 *et al.* 1996; Ogawa *et al.* 1997), hamster (Ogawa *et al.* 1999a), rabbit and dog

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Declaration of Interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

(Dobrinski *et al.* 1999a), primate (Nagano *et al.* 2001b), bull (Oatley *et al.* 2002) and cat (Kim *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 *et al.* 2003; Honaramooz *et al.* 2008) and pig (Honaramooz *et al.* 2002). Such transplantation between individuals from the same species 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 *et al.* 2003).

Two techniques have been widely used to reduce or deplete endogenous male germ cells in recipients: focal irradiation (Schlatt *et al.* 2002; Izadyar *et al.* 2003) and chemotherapeutic drugs (Ogawa *et al.* 1997; Brinster *et al.* 2003). 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 *et al.* 1997)], or into the rete testis [large animal models (Honaramooz *et al.* 2002; Honaramooz *et al.* 2003; Izadyar *et al.* 2003; Herrid *et al.* 2006)]. An additional concern in SSCT is the potential for rejection of the introduced cells by the recipient's immune system. To overcome this problem in mice, matching the strains of donors and recipients can be performed (Brinster and Zimmermann 1994); alternatively, one can induce immunological tolerance in the recipients (Kanatsu-Shinohara *et al.* 2003). In xenogeneic transplantation, in which cells from different donor species were transplanted into mice, immunodeficient mice were used to avoid rejection (Clouthier *et al.* 1996; Dobrinski *et al.* 1999a; Ogawa *et al.* 1999b; Dobrinski *et al.* 2000). 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 (Honaramooz *et al.* 2002; Honaramooz *et al.* 2003; Herrid *et al.* 2006). 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 *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 *et al.* 2005; Fujita *et al.* 2006) or wildlife conservation (Pukazhenthi *et al.* 2006). From a basic scientific perspective, SSCT can be used to investigate the fundamental characteristics of SSC (Parreira *et al.* 1998; Nagano *et al.* 1999; Kubota *et al.* 2003; Kent Hamra *et al.* 2005). Furthermore, because the male germline can be manipulated in the SSC prior to transplantation, SSCT can be used to generate transgenic animals (Nagano *et al.* 2001a; Kanatsu-Shinohara *et al.* 2006; Ryu *et al.* 2007; Honaramooz *et al.* 2008).

Over 360 naturally-occurring canine genetic diseases have been shown to have counterparts in humans, including various forms of cancer (Lingaas *et al.* 2003), blindness (Acland *et al.* 2005), and orthopedic defects (Athanasίου *et al.* 1995). Moreover, diseases such as muscular dystrophy and bleeding disorders (Tsai *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 the testes of five 3–6 month old donors 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 *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 the testes of a donor by sequential enzymatic digestions, first using collagenase then trypsin (Kim *et al.* 2006). After washing, the cell pellet was resuspended in “collection medium,” which contained Dulbecco's Modified Eagle's Media (DMEM), 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. Two million cells were placed into 0.1% poly-L-lysine-coated T75 cell culture flasks (Sigma), and maintained at 37°C in a

humidified 5% CO₂ atmosphere. 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. The cultured cells were harvested with 0.25 % Trypsin-EDTA, followed by washing with the culture medium. The cells were resuspended in the culture medium 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 (Aloka 633, Colormetrics Medical Systems Inc., Wallingford, CT) as previously described (Honaramooz *et al.* 2003), with minor modifications. An intravenous catheter (22G × 1 inch) was inserted through the caudal pole of the testis into the rete testis. To secure the position of the catheter during the course of the injection, a small drop of tissue glue (Nexaband S/C, Webster Veterinary Supply Inc., Sterling, MA), was applied to the skin at the injection site. Approximately 1 ml of cell suspension was gradually allowed to feed 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 Bouin's solution and processed for histologic examination.

Genotyping of the recipient sperm

As controls, donor and recipient genomic DNA were extracted from tunica vaginalis tissue and blood, respectively, using QIAamp® DNA mini-kit (Qiagen Inc., Valencia, CA). The sperm collected from the recipients 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) 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).

The technical approach used to quantify the percentage of donor-derived sperm was adapted from the manuscript of Niederstätter, *et al.* (Niederstätter *et al.* 2006). 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, ranging from 4×10^2 to 4×10^6 copy numbers, 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 × 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 Table 1 and Table 2, respectively.

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 *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 (Fig. 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. For these 3 recipients, the culture medium was used to suspend the cells for the injection. As the dogs were 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. Motility of epididymal sperm collected from these individuals was uniformly poor, visually assessed to be less than 5% (Table 3).

The other 2 dogs were transplanted with fresh mixed tubule cells, in the collection medium (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 (Table 3).

Histological sections revealed that 5% of seminiferous tubules showed evidence of spermatogenesis in the dogs transplanted with cultured cells, whereas 15% of the tubules showed evidence of spermatogenesis in the dogs transplanted with fresh cells. In both cases, the remaining tubules showed a Sertoli cell-only phenotype (Fig. 2). 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; Table 3). Furthermore, there were no apparent changes in the histological appearance of Leydig cells or Sertoli cells.

Genotyping of sperm collected from the recipients

Six microsatellite markers from the ‘Microsatellite Multiplex Set-2’ (Clark *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 (Fig. 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 4. 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).

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 (Lindblad-Toh *et al.* 2005; Khanna *et al.* 2006). 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 line of cells showing many of the critical characteristics of canine embryonic stem cells has recently been reported, but this line has not yet been shown to contribute to the germline (Hayes *et al.* 2008). A few reports have been generated from one group about cloning dogs by somatic cell nuclear transfer (Lee *et al.* 2005; Jang *et al.* 2007; Jang *et al.* 2008). Both of these approaches would provide other mechanisms 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 (Nagano *et al.* 2001a; Ryu *et al.* 2007; Honaramooz *et al.* 2008). 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 *et al.* 2006), in terms of electron energy and collimation cone diameter, to accommodate the larger testis size. 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. In addition to promoting normal reproductive behaviors, reducing sub-lethal damage to the somatic compartments might also improve the yield of donor spermatogenesis, based on evidence in rodents that somatic cell damage limits spermatogonial differentiation (Zhang *et al.* 2007).

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 component (likely additional amino acids and/or GDNF) of the culture medium for those cells 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 *et al.* 2002), goats (Honaramooz *et al.* 2003; Honaramooz *et al.* 2008), and bulls (Herrid *et al.* 2006). It should

be noted that additional experimentation beyond the scope of the current report would be required to resolve whether induction of immunological suppression or tolerance in the recipients might improve the efficacy of the SSCT procedure.

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 *et al.* 1999b; Honaramooz *et al.* 2002; Honaramooz *et al.* 2003; Herrid *et al.* 2006). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Leslie D. Appel and Marla Hirsch of Shelter Outreach Services, Ithaca, NY for providing testis specimens from routine castrations. We also thank Dr. Karin Hoelzer (Cornell University, Ithaca, NY) for her advice on the statistical analysis, and Mary Lou Norman for her help with histological processing.

Funding

This research was supported in part by an Empire State Stem Cell Board grant through the New York State Department of Health (to A.J.T.), the Baker Institute for Animal Health, and NIH HD-045664 (to A.J.T.).

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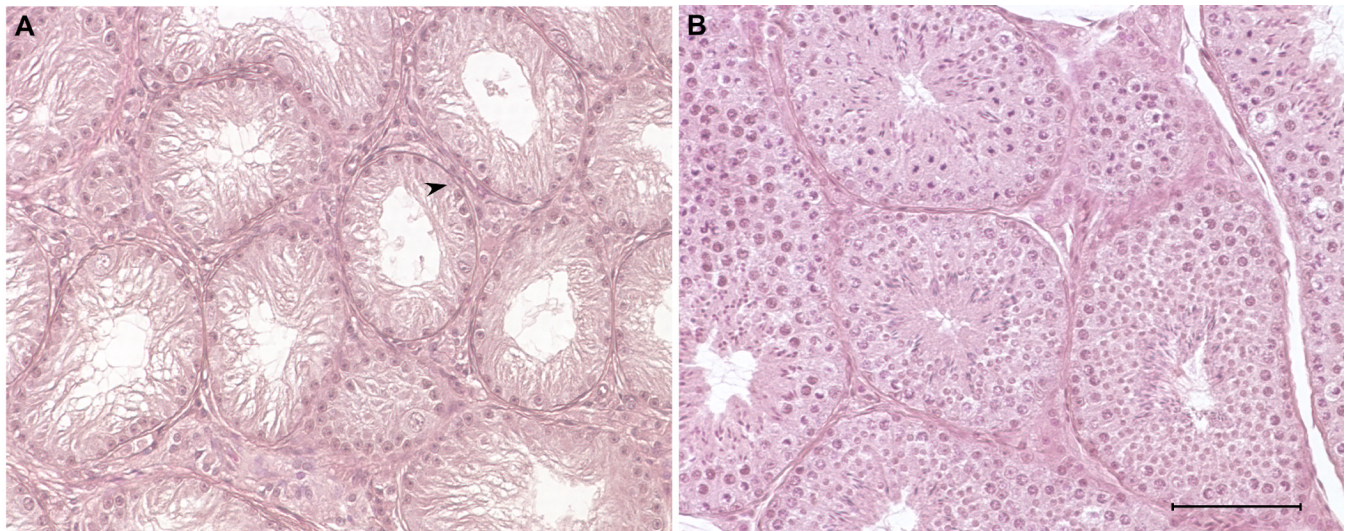


Figure 1.

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 μ m.

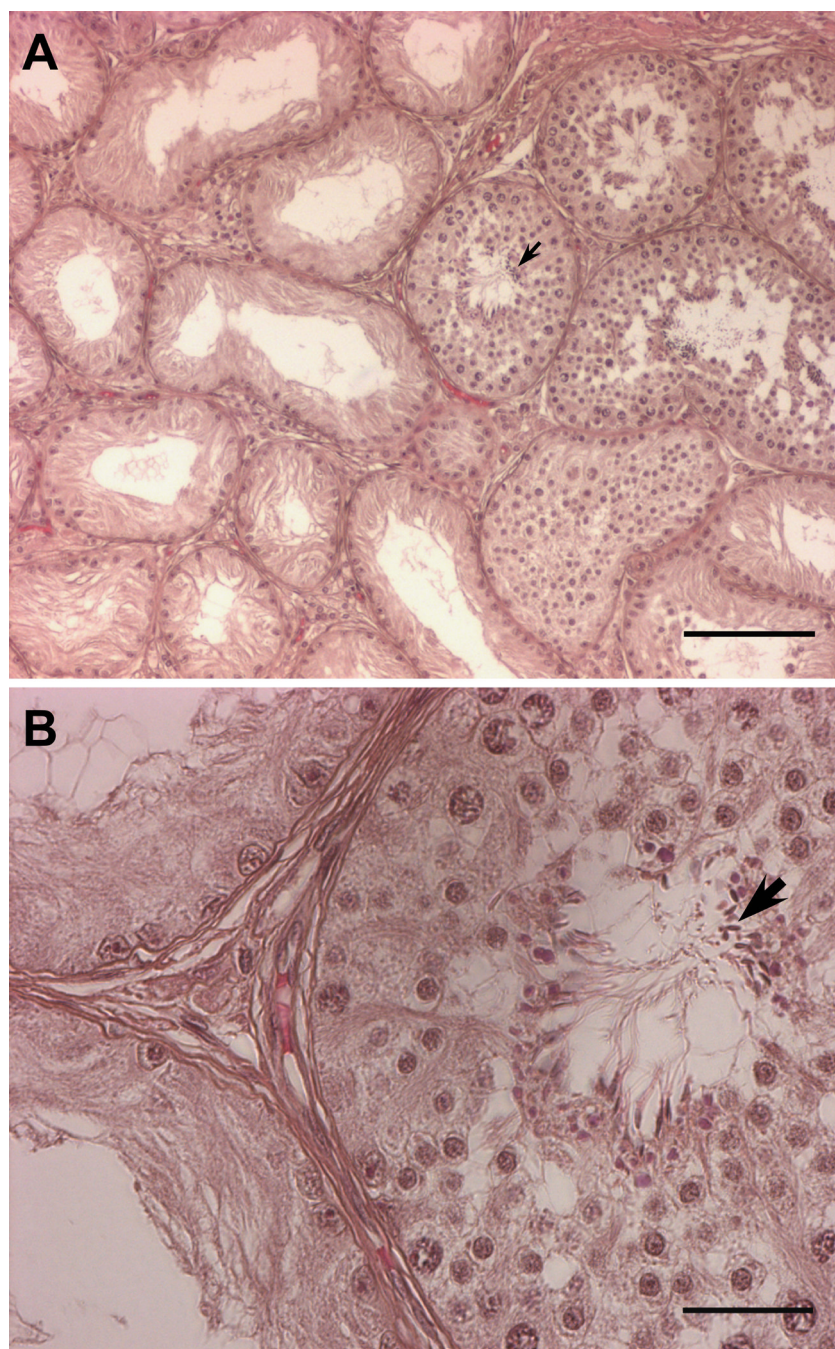


Figure 2. Cross sections of seminiferous tubules after SSCT with freshly isolated cells. Panel A shows both seminiferous tubules that have only Sertoli cells, and tubules that show full spermatogenesis (bar = 170 μ m). Panel B is a higher magnification view of panel A (Bar = 30 μ m). The arrows indicate the same spermatozoa in both panels.

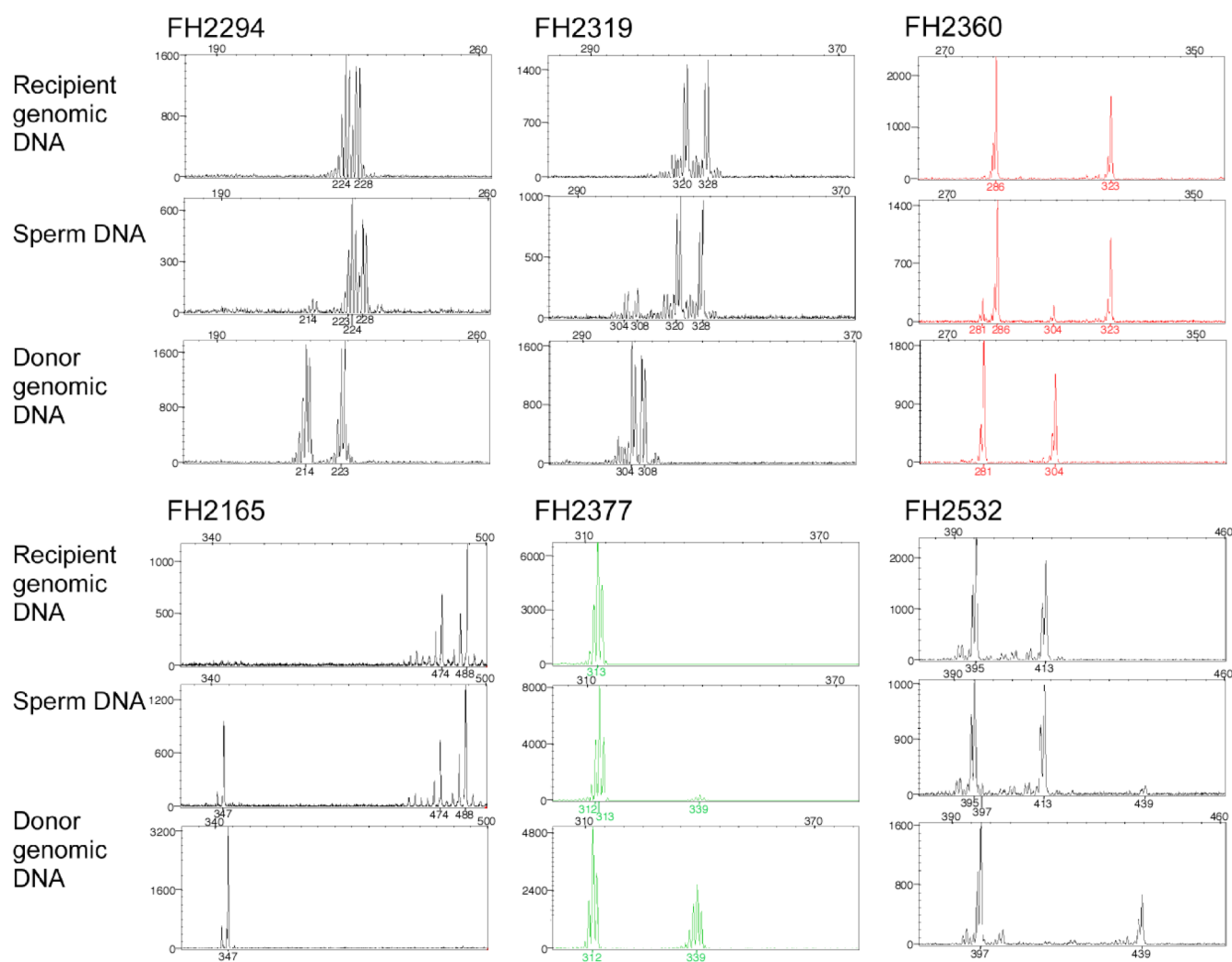


Figure 3. Genotyping plots of microsatellite analysis. There are three plots for each marker, with the name of each marker indicated on the top of the first plot. The plots were obtained with recipient genomic DNA, DNA from sperm produced by the recipient 12 months after SSCT, and donor genomic DNA, 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.