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

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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 four age groups of domestic cats (*Felis catus*; age range 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 spermatozoa 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 of age degenerated. Seminal vesicle weight, an indicator of bioactive testosterone, was not significantly different between donors aged 8 weeks to 7 months and controls, suggesting that xenograft Leydig cells were ultimately functional even in the 5–7 month age group. Regardless of donor age, production of mature spermatozoa from xenografts was markedly delayed compared with controls. Comparison of xenografts that produced sperm with normal controls revealed a decrease in tubule cross-sections having post-meiotic germ cells. Together, these results indicate that the maximum practical donor age was just before the onset of puberty and that even successful xenografts had abnormalities in spermatogenesis.

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 able to preserve genetic information from individual males has been the focus of much research (Pukazhenthi *et al*. 2006). Historically, considerable 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 towards the periphery of the seminiferous cords and transform into spermatogonia on the basement membrane (Bellve *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 *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, the use of these cells 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 spermatozoa 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 colonise and give rise to functional spermatozoa in several species, including mice (Brinster and Avarbock 1994; Ogawa *et al*. 2000), rats (Ryu *et al*. 2003) and goats (Honaramooz *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 *et al*. 1998; Shinohara *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 *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 optimised for suitability as recipients (Kim *et al*. 2006).

Xenografting of testis tissue is another technique that relies on the attributes of SSC to preserve male genetic information, but which preserves the architecture of the donor testis. This

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technique is performed by transplanting millimetre-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 *et al*. 2002), hamster and monkey (Schlatt *et al*. 2002), cattle (Oatley *et al*. 2005; Rathi *et al*. 2005), rabbits (Shinohara *et al*. 2002), cats (Snedaker *et al*. 2004), humans (Schlatt *et al*. 2006) and horses (Rathi *et al*. 2006).

Interestingly, the nature of xenograft spermatogenesis varies tremendously between species, with xenografted tissue showing a decreased time to spermatozoa production in the pig (Honaramooz *et al*. 2002) and primate (Honaramooz *et al*. 2004), no difference in timing of spermatozoa production in the bull (Oatley *et al*. 2004; Rathi *et al*. 2005) and remarkably delayed spermatozoa production in the cat (Snedaker *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 may have effects on the ability of xenografts to support spermatogenesis. In most species, full spermatogenesis can be observed in xenografts from neonatal donors (Honaramooz *et al*. 2002; Schlatt *et al*. 2003; Snedaker *et al*. 2004; Oatley *et al*. 2005), whereas limited attempts with fully adult testicular tissue have not supported spermatogenesis (Geens *et al*. 2006; Rathi *et al*. 2006; Schlatt *et al*. 2006). Beyond this broad division, only two studies have examined the effect of donor age on xenograft success. Of these, work performed in cattle focused exclusively on early prepubertal donors (Schmidt *et al*. 2006) and work performed on stallions primarily investigated the endocrine regulation of xenograft function (Rathi *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. Our results provide an effective age range for donors, describe further the nature of the delay in spermatozoa production in felid testis xenografts (which sets the cat apart from other animal models studied to date) and give insight into how donor age affects testis xenograft success.

Materials and methods

Reagents

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

Animals and xenografting procedure

Donor testes from domestic cats (*Felis catus*; $n = 12$) were obtained from routine castrations at local veterinary hospitals and shelters. Because of the age range of donor tissue used in a previous study (Snedaker *et al*. 2004), we concentrated our efforts on the use of donors greater than 5 weeks of age. In our preliminary experiments, testis xenografts from fully adult donors (older than 1.5 years) uniformly degenerated (data not shown). We therefore narrowed our study to four age groups: (*1*) 6–8 weeks old (weaning–early juvenile); (*2*) 9–16 weeks old (prepubertal); (*3*) 5–7 months old (pubertal); and (*4*) 8–15 months old (young adult). In practice, all donors for the youngest group were 8 weeks of age, refining our definition of that group. These age groupings clearly distinguish the prepubertal from pubertal, because it has been shown that the first spermatozoa can be found within testes between the 5th and 7th months (Tsutsui *et al*. 2004). For the present study to have any use, the origin of any sperm found within a xenograft must be clear regarding whether produced before or after xenotransplantation. All samples were stored and transported in sterile saline at 4°C and used within 24 h after collection. Testes were washed in cold phosphate-buffered saline (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 mm^3. These specimens were kept in DMEM containing 100 $\mu g\,mL^{-1}$ streptomycin sulfate and 100 $IU\,mL^{-1}$ penicillin on ice until grafting. Four to 8-week-old ICR/SCID mice (Taconic, Germantown, NY, USA) were used as recipients. Anaesthesia 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 eight to 10 xenografts were placed under the skin (four to five 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, USA) 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, Braintree, MA, USA). At the end of surgery, buprenorphine (1 $mg\,kg^{-1}$) 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 can be found in Table 1.

Analysis of xenografts

Based on the findings of Snedaker *et al*. (2004), we evaluated the progress of spermatogenesis at 10, 30 and 50 weeks after xenograft placement. At these times, two or more xenografts from multiple donors were retrieved from recipient mice. The xenografts collected 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 before being embedded in paraffin and sectioned at 4 μm. After mounting on slides, each section was deparaffinised with xylene

Table 1. Experimental design for evaluating the effect of donor age on spermatogenesis in domestic cat testis xenografts

Age group	No. donors[A]	Donor age	No. mice[B]	Total grafts[C]
8 weeks	3	8 weeks	2	49
		8 weeks	2	
		8 weeks	2	
9–16 weeks	3	10 weeks	2	50
		14 weeks	2	
		16 weeks	2	
5–7 months	3	5 months	1	36
		5 months	1	
		7 months	2	
8–15 months	3	8 months	2	51
		12 months	2	
		15 months	2	

[A]Total number of donors for each age group.
[B]Number of recipient mice used per donor.
[C]Total number of xenografts placed for each age group.

and hydrated with 100% ethanol, then with 70% ethanol and finally with water before staining with haematoxylin and eosin. Sections were scanned under an Eclipse TE2000-U microscope (Nikon, Melville, NY, USA) and images were captured using a Retiga 1300 colour camera (QImaging, Burnaby, BC, Canada) or a Spot RT camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Xenograft cross-sections were evaluated for the appearance and architecture of seminiferous tubules and Leydig cells, as well as 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 emphasises the production of later stages of spermatogenesis because the primary goal of this work is to produce elongating spermatids and mature spermatozoa to be used for technologies of assisted reproduction. At least 10 histological sections from 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 spermatozoa 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.

Statistical analysis

All data were analysed using Origin 7.0 Software (OriginLab, Northampton, MA, USA). An 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, Student's *t*-test was performed to compare differences in spermatogenic cell development between xenografts and normal cat testes. Statistical significance was considered at $P < 0.05$.

Results

Histological analysis

Table 2 shows that the percentage of xenografts recovered from recipient mice decreased as donor age increased and that the percentage of the xenografts containing germ cells at different stages of spermatogenesis varied with both donor age and the retrieval time points. For the 8- and 9–16-week-old donor age groups in our study, we had a percentage recovery rate very similar to that reported for other species (Rathi *et al*. 2005; Zeng *et al*. 2006).

At the 10 week time point, spermatocytes were seen as the most advanced germ cell stage in successful xenografts from the three youngest donor groups (i.e. 8 and 9–16 weeks and 5–7 months old; Fig. 1*b*, *f*, *j*, respectively). Seminiferous tubules in xenografts collected at this time from 8–15-month-old donors had degenerated (Fig. 1*n*). Early elongating spermatids were the most advanced germ cells in the xenografts of 9–16-week-old donors (Fig. 1*g*) at 30 weeks after xenografting, whereas spermatocytes were the most advanced germ cells in xenografts from the 8-week-old (Fig. 1*c*) and 5–7-month-old groups (Fig. 1*k*) 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 (Fig. 1*o*). Complete spermatogenesis, defined by the presence of mature testicular spermatozoa, was observed in 13.3% and 25.0% of recovered xenografts from the 8- (Fig. 1*d*) and 9–16-week-old groups (Fig. 1*h*), respectively, at 50 weeks after the procedure. Elongating spermatids were found as the most advanced stage of germ cell development from one 7-month-old donor (Fig. 1*l*), whereas xenografts from donors 8 months of age or older were invariably degenerated at 50 weeks after implantation (Fig. 1*p*).

Evaluation of xenograft testosterone production

Seminal vesicle development is highly androgen dependent (Setchell 1978). Therefore, in the castrated recipients, seminal vesicle development is commonly used as a marker for xenograft production of bioactive testosterone (Schlatt *et al*. 2003; Rathi *et al*. 2006; Schmidt *et al*. 2006). The average weight of recipient seminal vesicles varied only slightly between the three youngest donor age groups (174.2 ± 41.2, 157.3 ± 53.7 and 215.0 ± 29.2 mg for 8- and 9–16-week-old and 5–7-month-old donors, respectively; Fig. 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

Table 2. Effect of donor age on recovery and parameters of spermatogenesis in feline testis xenografts

Age group	% Total grafts recovered[A]	Time after grafting (weeks)	No. grafts retrieved per time point	Percentage of xenografts containing different stages of male germ cell development[B]				
				SG	SC	RS	ES	Sperm
8 weeks old	81.6	10	10	90.0	30.0	–	–	–
		30	15	73.3	33.3	–	–	–
		50	15	80.0	80.0	40.0	20.0	13.3
9–16 weeks old	72.0	10	5	80.0	80.0	–	–	–
		30	7	100	100	57.1	28.6	–
		50	24	100	100	70.8	50.0	16.6
5–7 months old	63.8	10	8	50.0	25.0	–	–	–
		30	7	42.8	28.5	–	–	–
		50	8	87.5	50.0	25.0	12.5	–
8–15 months old	33.0	10	6	–	–	–	–	–
		30	11	9.0	9.0[C]	–	–	–
		50	0	–	–	–	–	–

[A]Total number of xenografts retrieved divided by the total number of grafts placed.
[B]Total number of xenografts that contained the indicated stage of germ cell development divided by the total number of xenografts retrieved at that time point.
[C]The spermatocytes in this section did not appear to be normal.
SG, spermatogonia; SC, spermatocytes; RS, round spermatids; ES, elongating spermatids; sperm, spermatozoa.

mice (294.8 ± 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 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 *v*. 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 (Fig. 3*a*, *b*), 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 (Fig. 3*b*). 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 (Fig. 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 (Figs 4*a*, 5*a*). Data in Fig. 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-sections, including those containing no germ cells. To compare spermatogenesis in the xenografts with that in normal cat testes more accurately, we also determined percentages after having factored out the empty, degenerated tubules (Group B). Even with this correction, a significant difference ($P < 0.001$) was observed in the pattern of spermatogenesis between tubules in xenografts compared with normal testicular tissue from young adult cats ($n = 3$). The percentage of tubules containing pachytene spermatocytes and elongating spermatids was not significantly different, but the percentage containing round spermatids and spermatozoa was 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.

Discussion

Ectopic testis xenografting has been performed using many different species as donors (Honaramooz *et al*. 2002; Schlatt *et al*. 2002, 2006; Shinohara *et al*. 2002; Snedaker *et al*. 2004; Oatley *et al*. 2005; Rathi *et al*. 2006). However, as noted above, there have been great discrepancies between species in terms of the timing of xenograft spermatozoa production *v*. that seen under normal physiological conditions. Of the species studied, the cat is noteworthy because of the marked delay until spermatozoa are produced (Snedaker *et al*. 2004). For this species, only one report has been published and this used a narrow donor age range (1–5 weeks; Snedaker *et al*. 2004). Across species, the variable of donor age has not been studied intensively, 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.

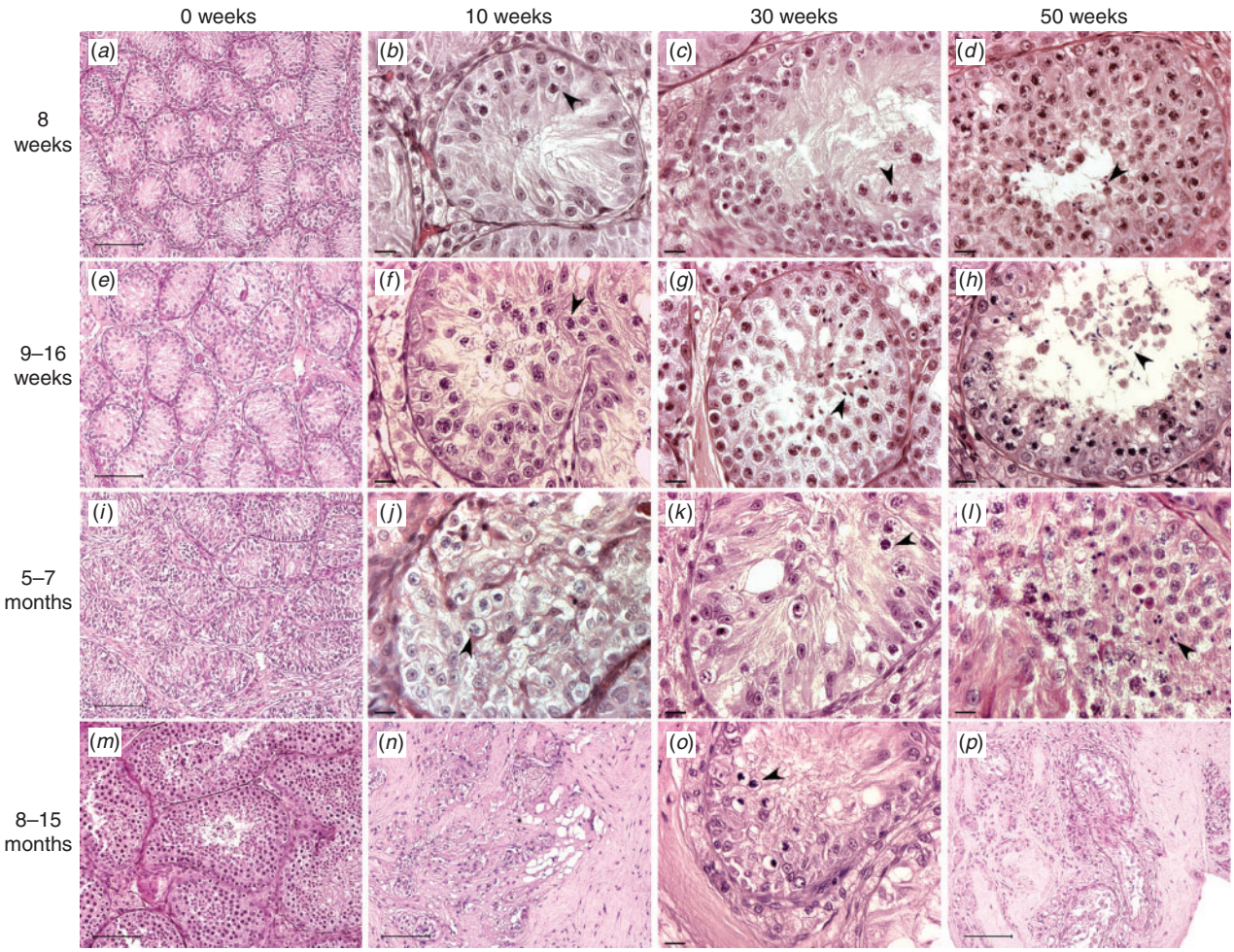

Fig. 1. Cross-sections of xenograft seminiferous tubules. Figure parts shown under '0 weeks' are representative of the histological appearance of donor tissue at the time of xenografting. (*a*) An 8-week-old donor; (*e*) a 14-week-old donor; (*i*) a 5-month-old donor; (*m*) a 12-month-old donor. Xenografts were collected from the different donor age groups, namely 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 after the procedure. Spermatocytes (arrowheads in parts *b*, *c*, *f*, *j*, *k* and *o*) were the most advanced cells from the xenografts collected at 10 (*b*, *f*, *j*) and 30 (*c*, *k*, *o*) weeks after grafting. Elongating spermatids (arrowheads in parts *g* and *l*) were the most advanced germ cells from the 9- to 16-week-old donor xenografts collected at 30 weeks (*g*) and from the 5- to 7-month-old donor xenografts collected at 50 weeks (*l*) after the procedure. Arrowheads in parts *d* and *h* indicate spermatozoa as the most advanced germ cells in the xenografts collected at 50 weeks for the 8- and 9- to 16-week-old donor age groups. Xenografts from young adult donors were all degenerated at 10 and 50 weeks after xenografting (parts *n* and *p*, respectively), although apparently abnormal spermatocytes were seen rarely from one donor at the 30 week time point. Bar = 15 μm (except for parts *a*, *e*, *i*, *m*, *n* and *p*: 100 μm).

A major finding of the present 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: (*1*) for donors ≥8 months in age, there was complete degeneration of xenografts; and (*2*) for donors aged 5–7 months, the period when the first sperm are produced and spermatogenesis begins in most tubules (Tsutsui *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-old group and of elongating spermatids from one donor in the 5–7-month-old 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 spermatozoa in epididymides from 5-month-old cats, although no histological evidence for complete spermatogenesis was seen in most testis cross-sections examined from those animals (Kim *et al*. 2006). Conversely, the one 7-month-old donor may 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 with age-matched controls (compare Fig. 1*b* and *i*). Spermatocytes were observed in approximately one-third of the xenografts

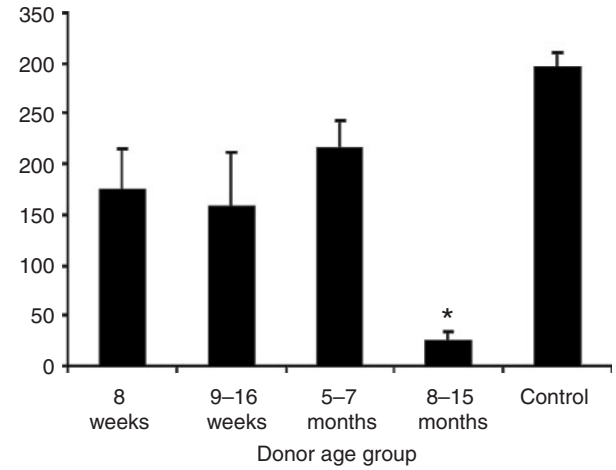

Fig. 2. Seminal vesicle weight for each donor age group. Seminal vesicles were collected and weighed at 50 weeks after xenografting and were compared against control tissue from intact retired breeder SCID mice ($n = 4$). Data are presented as mean ± s.e.m. $*P < 0.05$ between the 8–15-month-old age group and the control.

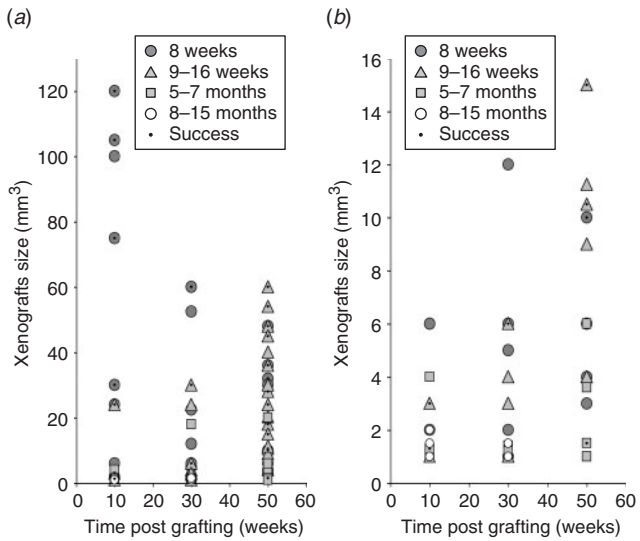

Fig. 3. Scatter plot of xenograft size and outcome. Xenografts were collected from each donor group at three different time points after the procedure and measured. (*b*) Close-up view of smaller-sized xenografts (0–16 mm^3) from (*a*) to show that small size did not necessarily preclude success.

10 weeks after the 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 *et al*. 2005; Rathi *et al*. 2005; Zeng *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 *et al*. 2002; Schlatt *et al*. 2003; Rathi *et al*. 2005).

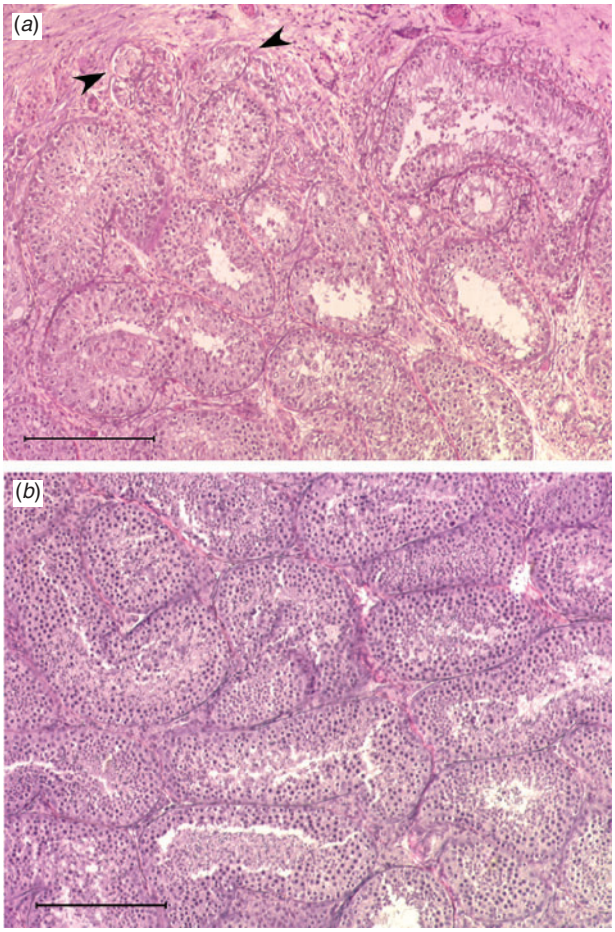

Fig. 4. Cross-sections of a successful testis xenograft and a control testis. (*a*) A xenograft at 50 weeks after the procedure from the 8-week-old donor group. Note the appearance of degenerating tubules (arrowheads) interspersed between tubule cross-sections containing meiotic germ cells. Note also the connective tissue capsule that formed around the xenografts. (*b*) A 12-month-old control. Bar = 500 μm.

Despite this initial hastening, fully mature spermatozoa 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 spermatozoa 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 may 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-old donor group, spermatogenesis was initially slightly delayed compared with age-matched control testis,

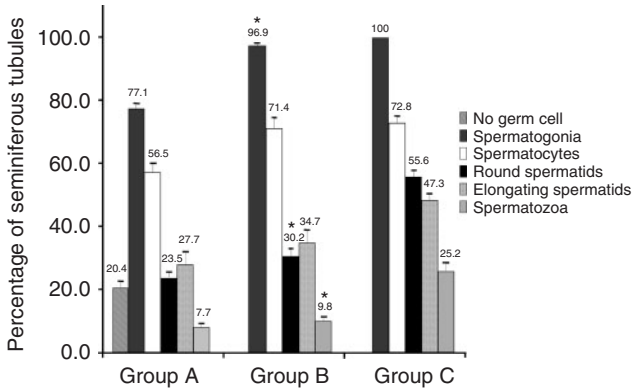

Fig. 5. Percentage of seminiferous tubule cross-sections containing different stages of spermatogenic cells. Percentages in Groups A and B were derived from three xenografts that produced morphologically mature sperm at the 50 week time point, and percentages in Group C were derived from testes of three 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 Group A, but the number of tubule cross-sections containing no germ cells was excluded from the total ($n = 1184$). In the control Group C, the number of tubule cross-sections containing each germ cell type was divided by the total number of cross-sections assessed ($n = 900$). Data are presented as mean ± s.e.m. $^*P < 0.05$ between Groups B and C.

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 may have found mature spermatozoa in some xenografts earlier than 50 weeks. However, our experimental design was derived to maximise the chance of finding success, as opposed to finding the earliest time of xenograft success.

Differences in xenograft success between prepubertal and pubertal donor testes offer the possibility that inherent differences in the stem cell cohorts between these ages may contribute to success or failure in the 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 *et al*. (2005) suggested that meiotic/post-meiotic cells may 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 (Grootegoed *et al*. 1986; Nakamura *et al*. 1986; Bajpai *et al*. 1998) 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, have been the same as in prepubertal 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 that affected the ability to recover from this initial loss. One potential endocrine-based rationale may 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 spermatozoa 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 spermatozoa could have a significant impact on the 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 may depend on the timing of the spermatogenic wave within that region. To compare xenograft spermatogenesis more accurately with normal controls, we factored out those cross-sections that did not support male germ cell development. Still, the remaining tubule cross-sections showed differences compared with 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 towards other changes in meiosis that were not detectable histologically is an extremely important question. Additional research on the quality and function of spermatozoa produced by testis xenografts is an essential next step before clinical utilisation. The present findings suggest that this technique indeed represents a potentially important complement to current conservation efforts by being useful for the age range of neonatal to prepubertal males. 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. Testis xenografting offers a means of preserving the breeding potential of individuals too young to produce mature spermatozoa.

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