Successful transplantation of bovine testicular cells to heterologous recipients

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Abstract

While heterologous germ cell transplantation was successful in pigs and goats, autologous transplantation alone has been reported to result in donor-derived spermatogenesis in cattle. The objective of this study was to investigate whether the transplantation of heterologous germ cells could result in colonization of recipient testes in cattle of different breeds. Testicular cells were isolated from 8 *Bos taurus* donor bull calves and then transferred into 15 *Bos indicus*-cross bull calves. All animals were pre-pubertal, donors were aged 5–7 months and recipients 5–11 months, and scrotal circumferences ranged from 15 to 22 cm. Single cell suspensions of donor testicular cells, prepared by enzymatic digestion, were labelled with fluorescent dyes PKH26 or CFDA-SE, before transfer into the rete testis of recipients under ultrasonographic guidance. To assess the longevity of colonization by donor cells, recipients were castrated 2–30 weeks after cell transfer. Donor cells were observed in 15/25 (60%) of the testes that received PKH26-labelled cells, whereas no CFDA-SE-positive cell was identified in any recipients. The maturity of the donors or recipients (measured by scrotal circumference) did not affect colonization potential. In freshly isolated tubules, clumps of PKH26-positive cells were observed, which indicated either cell division or extensive local colonization of specific areas of the tubules. In frozen sections, PKH26-positive cells were identified on the seminiferous tubule basement membrane, which indicated that these cells had successfully migrated from the tubule lumen and were likely to be spermatogonia. We conclude that PKH26 was more suitable for labelling donor testis cells and donor cells can be identified up to 6 months following transfer. These results indicate that allogeneic transplantation of testicular cells can occur between *Bos taurus* and *Bos indicus* cattle. Further studies will investigate functionality of transferred testicular cells.

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Introduction

Male germ cell transfer is commonly used in rodents to study the control of spermatogenesis with the ultimate goal to enhance or suppress male fertility. The transplantation technique uses testis stem cells, harvested from a donor animal, then transferred into recipient testes (Brinster & Zimmermann 1994, Jiang & Short 1995). The technique's success in mice and rats raised the possibility of its use as an alternative method of transgenesis in livestock animals and recently offspring were produced from germ cell transfers in goats (Honaramooz *et al.* 2003b).

The technique used in goats and pigs is readily adapted to cattle (Honaramooz *et al.* 2002b, 2003a,b, Izadyar *et al.* 2003, Joerg *et al.* 2003). In cattle, germ cell transfer has the potential to use in commercial breeding systems. By transplanting stem cells from elite bulls into

lesser males followed by natural service, elite genetics could be disseminated more widely. The large scale culture of the donor germ line stem cells would provide a renewable source of stem cells for transplantation. This system would create an alternative to artificial insemination (AI) for the use of elite sires in the cattle industry in areas where AI is not practicable (Hill & Dobrinski 2006). Although AI is the most commonly used breeding technique for this purpose, its use in extensive beef cattle grazing systems is limited by the logistics involved in handling cattle during the AI procedures. In these extensive areas, the germ cell transplant technique could fill a niche in beef cattle operations by facilitating crossbreeding or introduction of new genetics via natural service.

The present experiments were designed to test the potential of germ cell transplant in beef cattle under field conditions, using commercial cattle of different breeds.

We have investigated three factors integral to the evaluation of the germ cell transplant (GCT) technique.

Recipient characteristics were investigated through the use of unprepared or selected recipients. These recipients have neither been depleted of endogenous testis stem cells prior to transplant, nor were they immune suppressed or pre-selected for similar tissue type prior to transplant. In rodents, successful germ cell transplantation requires recipients to be either immunosuppressed or closely related to the donor animal (Brinster & Zimmermann 1994, Jiang & Short 1995, Kanatsu-Shinohara *et al.* 2003, Zhang *et al.* 2003). In goats, a kid of donor cell origin was produced from the sperm produced by a randomly selected recipient that did not undergo stem cell depletion prior to transplant of testicular germ cells (Honaramooz *et al.* 2003b). In cattle, autologous transplantation has resulted in colonization of seminiferous tubules, while transplantation of testicular cells from a heterologous donor has not (Izadyar *et al.* 2003). In the present study, we test the outcome of transplants into non-depleted recipients of a different breed to the donor animal.

The maturity status (relative to onset of spermatogenesis) of donor and recipient testes was investigated. This involved the use of donors and recipients prior to and after the onset of spermatogenesis. As testis maturity of the donor or recipient may affect the outcome of the transplantation procedure, we have selected animals at tubule developmental stages just prior to and during the onset of active spermatogenesis. In the mouse, spermatogenesis starts a few hours after birth and the number of spermatogonia increases to maximal levels by day 21 (Baker & O'Shaughnessy 2001). However, in bulls, the onset of spermatogenesis is delayed until several months after birth and development varies between breeds. In the Fleckvien breed, the prespermatogonia are still in a phase of relative mitotic quiescence until the 4th postnatal week, and expansion of spermatogonia reaches a maximum between 4½ and 8 months of age (Wrobel 2000). However, in pure Brahman bulls, spermatogenesis starts at 9 months (Aponte *et al.* 2005). The rapid proliferation of germ cells and Sertoli cells during the onset of spermatogenesis leads to an increase in tubular diameter and testis size, which can be measured indirectly by changes in the scrotal circumference (SC) (Lunstra *et al.* 1978, Wrobel 2000).

The longevity of donor cells after transplant into recipient testes was assessed using two marker dyes. This also illustrated the duration of dye detectability in bovine testis cells. Cell tracking dyes are commonly used in immunological studies to track lymphocyte migration and proliferation up to 6 months (Parish 1999). We selected two dyes (PKH26 and CFDA) for comparison with a goal of determining the most cost effective and reliable method of assessing short-term outcome of the germ cell transplant procedure. Previously, PKH26 has been demonstrated to label testis cells in goats and

pigs up to 3 months following germ cell transplant (Honaramooz *et al.* 2002a,b, 2003a).

This manuscript describes these initial steps required to assess the application of the male germ cell transplant technique as a cattle breeding system.

Materials and Methods

Animals

Donor animals used in this experiment were 8 *Bos taurus* (5–7 month old Murray Grey) bull calves. At castration, their SC varied from 18 to 22 cm. Recipient animals were 15 *Bos indicus* (5–11 month old Brahman-cross) bull calves with SC ranging from 15 to 21 cm at the time of testis cell transfer. Animals were handled and treated according to the guidelines of the Animal Ethics Committee at CSIRO, Armidale.

Collection of testes and histology

Under general anaesthesia with a combination of Xylazil (0.1 mg/kg) and Ketamine (3 mg/kg), eight donor calves were castrated. The body weights and SC were recorded during castration and the data were used for the analysis of correlation to the testicular maturity. The testes were surgically removed and transferred on ice to the laboratory within 1 h. Under sterile condition, the tunica albuginea were removed from the testes and washed with Dulbecco's PBS (DPBS) supplemented with antibiotics. The testes were placed in sterile plastic bag with DPBS and stored at 4 °C overnight. On the day of isolation, paired testes were trimmed of epididymides and excess connective tissue, and weighed. A small piece of testicular sample (about 1 cm³) was collected from equatorial region of both the testes in each animal and fixed in Bouin's solution and embedded in paraffin wax. Sections (5 µm) were stained with haematoxylin. The identification of cell types was based on the criteria as described (Russell *et al.* 1990). The most advanced germ cell types were counted in 100 round cross-sections from both testes under a light microscope to evaluate testis maturity status. The tubular diameter and presence of a visible lumen were also recorded in those tubules.

Preparation of donor cells prior to transfer

The following morning, a two-step enzymatic isolation procedure was used to isolate individual tubular cells essentially as described by Honaramooz *et al.* (2002a,b). In brief, approximately 10 g testis tissue was dissected free of the rete testis and connective tissue. The tissue was washed thrice with DMEM with antibiotics for 10 min intervals and then incubated with collagenase (2 mg/ml, type 5; Sigma) at 37 °C for 40 min with occasional agitation. The supernatant was removed, and the remaining pellets containing testis fragments were further

digested with collagenase (2 mg/ml) and hyaluronidase (2 mg/ml) at 37 °C for 15 min. After being rinsed five times in DPBS without calcium and magnesium at room temperature, the fragments were then treated with trypsin (2.5 mg/ml; Gibco) in DPBS for 5–10 min at 37 °C. DNase I (7 mg/ml; Sigma) in DMEM was added 1 min after trypsin treatment. An equal volume of heat inactivated foetal bovine serum (FBS, Gibco) was used to inactivate trypsin digestion. The resultant cell suspension was then filtered through a cell strainer with two layers of nylon mesh (upper layer 96 µm and lower layer 55 µm pore size), centrifuged at $400\times g$ for 5 min at room temperature. The pellets were resuspended in 10 ml DMEM containing 5% FBS with the density $40\text{--}80\times 10^6$ cells/ml. The cell viability was assessed by trypan blue exclusion. The cell suspension was stored overnight in 175 cm² Falcon culture flasks (50×10^6 cells/flask) in DMEM + 5% FBS, at 37 °C in 5% CO₂, then the cells were recovered next morning to transfer into recipients. After overnight storage in flasks, non-adherent cells were collected for transfer by vigorous shaking of the plates.

Assessment of spermatogonia population

Spermatogonia were identified using protein gene product 9.5 antigen (PGP 9.5, Chemicon Australia Pty Ltd, Melbourne, Australia) in Bouin's fixed testis sections from the donor animals and also in the cell suspensions after overnight culture (Wrobel *et al.* 1995, Rathi *et al.* 2005). Slides were quenched by incubating in methanol-peroxide for 5 min to block the endogenous peroxidase activity, permeabilized with Tris-buffered saline tween-20 + 0.01% Triton X-100 for 5 min and blocked with 0.5% BSA in TBS for 30 min. Sections were incubated with polyclonal rabbit anti-PGP 9.5 antibody (Chemicon) at 1:2000 in the blocking medium overnight at 4 °C. This was followed by incubation with biotinylated secondary antibody (1:500, goat anti-rabbit immunoglobulin, Sigma) for 60 min at room temperature. All incubations were performed in a moist chamber. The results were visualized using the ABC system (Vector, Burlingame, CA, USA). Finally, the sections were counterstained with haematoxylin to visualize the tissue. Other steps were same as that of tissue staining, except fixing smears in 4% cold paraformaldehyde and incubating with primary antibody at 1:10 000.

Labelling of testicular cells and transplantation

Prior to transfer, cells were labelled with fluorescent dye to monitor their fate in the recipient testis. Two fluorescent dyes were used: Red Fluorescent Cell Linker (PKH26; Sigma) and Vybrant CFDA-SE cell tracer Kit (Invitrogen) according to manufacturers' recommendations.

For PKH26, 50 million cells in a 15 ml test tube were centrifuged at $400\times g$ for 5 min to form a loose pellet, and resuspended in 500 µl of the supplied diluent. At the same time, 40×10^{-6} M PKH26 dye was freshly prepared by mixing the stock with diluent C. Five hundred microliters of PKH26 dye were added into the cell suspension and gently mixed. After 3 min incubation, the reaction was terminated by addition of an equal volume of FBS. Cell pellets were transferred to a new tube and washed thrice with DMEM, then resuspended in DMEM + 5% FBS for transfer.

To stain cells with CFDA-SE, 50 million cells were washed once with DPBS and resuspended in 8 ml CFDA-SE solution with a concentration 25×10^{-6} M. The cell suspension was incubated 15 min in a 37 °C water bath. At the end of incubation, the cells were centrifuged at $400\times g$ for 5 min and resuspended in DMEM, and incubated at 37 °C for another 30 min. Cells were washed twice with DMEM and resuspended in DMEM + 5% FBS for transfer.

The proportion of cells labelled was similar for the two fluorescent dyes (>85%).

Transfer of testis cells into recipients

Transfer of testicular cell preparations into 15 recipients was performed under general anaesthesia via ultrasound guided injection into the rete testis using the technique described for pigs by Honaramooz *et al.* (2002a,b). After successful placement of a catheter in the rete testis, 5 ml cell suspension with an average concentration 25×10^6 cells/ml was injected over 1–2 min. To confirm a successful injection, a small quantity of air was injected into the testis after injection of cells to visualize the distribution of the injected fluid.

To directly compare the labelling efficiency of the two dyes, CFDA-SE-labelled cells were injected into one testis and PKH26-labelled cells were injected into the contralateral testis on the same day in three recipients. These animals were castrated 2, 4 and 8 weeks after transfer. In the remaining recipients, both testes were injected with cells labelled with CFDA-SE (1 recipient) or PKH26 (11 recipients) and these animals were castrated 2–30 weeks after transfer.

Overall, a total of 25 testes were injected with PKH26-labelled cells (14 recipients) and 5 testes were injected with CFDA-SE-labelled cells (4 recipients). Following transplant, recipients were treated with an antibiotic (Alamycin, 20 mg/kg) and anti-inflammatory (Flunixin, 2.2 mg/kg), then closely monitored for 3 days for reaction to the transplant procedure.

Detection of donor cells in recipient testes

At 2–30 weeks after transplantation, calves were castrated under general anaesthesia. Samples from these testes were prepared for analysis of labelled cells via wet mounts of

tubules, individual cell isolation and frozen sections. Each testis was evenly cut into five sections and three pieces of tissues were collected from each section for the following usage: 1 g tissue for germ cell isolation; 0.4–0.6 cm pieces tissues for wet mount tubules isolation and 0.8–1 cm pieces tissue for frozen samples. Tubule fragments were isolated and a single cell suspension was produced according to the methods outlined for donor cell preparation. In frozen sections, 100 tubules/slide were counted to localize the PKH and CFDA-SE-positive donor cells. All samples were examined under transmitted light and fluorescent microscopy (rhodamine and green filter) 100–400 $\times$ magnification.

Statistical analysis

Analyses were performed using the SAS System for Window, Release 8.01. Data were presented and expressed as mean $\pm$ S.E.M. Variations of individual points from baseline were investigated using NPAR1WAY Wilcoxon option and the Kruskal–Wallis chi-squared test. Correlations were performed using the correlation procedure of the SAS System. Differences were considered significant when the *P* value was <0.05 .

Results

Donor testis maturity

As the SC of donor animals varied (18–22 cm), tubule morphology was compared between the donors grouped into SC of 18–20 cm (testis weights 44–54 g, $n=4$) and 21–22 cm (66–88 g, $n=4$). The primary goal was to investigate a correlation between tubule maturity and outcome of the germ cell transplant procedure. Animals with an SC 18–20 cm, possessed less mature tubules than those with 21–22 cm as indicated by the most advanced germ cell type present in tubules: spermatogonia (45.3 ± 2.1 vs $13.7 \pm 1.2\%$ Fig. 1A); spermatocytes (40.1 ± 3.2 vs $30.2 \pm 2.3\%$ Fig. 1B); and spermatids (14.6 ± 3.5 vs $56 \pm 4.6\%$ Fig. 1C). Tubule diameters 80–120 μ m predominated ($86.4 \pm 6.8\%$) in animals with an SC 18–20 cm compared with 120–180 μ m ($68.3 \pm 8.6\%$) in animals with an SC 21–22 cm. The

tubule lumen was not visible in the majority of tubules from animals with an SC 18–20 cm ($80.2 \pm 4.8\%$) of testes, whereas the majority with an SC between 21 and 22 cm displayed a lumen ($53.4 \pm 4.3\%$).

Regression analysis revealed that testicular weight ($r=0.56$, $P<0.05$) and SC ($r=0.52$, $P<0.05$) were moderately correlated to tubule diameter and there was no significant correlation between body weight ($r=0.45$, $P>0.05$) and stage of tubule maturity ($r=0.43$, $P>0.05$).

Preparation of donor cells prior to transfer and assessment of spermatogonia population

A mean of 37 million cells were isolated per gram of testis tissue (range 22–66 million per gram). The viability of the cells after enzymatic isolation averaged 90% and decreased to 79% following overnight storage. Smears from cell suspensions contained $32 \pm 1.1\%$ cells that were positively stained with PGP 9.5 (Fig. 2B).

Sections from each of the eight donor testes were stained for PGP 9.5 and positive cells were found round with a spherical nucleus situated generally on the tubular basal lamina (Fig. 2A). The PGP 9.5 positive cells were often arranged in singles or pairs and were also occasionally observed in the centre of the tubules. Almost all tubules (95%) contained PGP 9.5 positive cells with mostly 3–4 PGP 9.5 positive cells per tubule.

Comparison of PKH26 and CFDA dyes for detection of donor cells in recipient testes

Three recipients injected with both CFDA-SE- and PKH26-labelled cells (one dye per testis) were castrated 2, 4 and 8 weeks following transplant. PKH26 was shown to be the preferred dye to track testis cells following transplant. PKH26-positive donor cells were found in each recipient testis, whereas no CFDA-SE-positive donor cell was identified in any of the recipients. In the PKH26-positive testes, clusters or chains of donor cells in multiple areas throughout seminiferous tubules were observed in isolated tubules (Fig. 3). PKH26-positive cells were found on the basement membrane as single cells in the cryosections (Fig. 3B). No CFDA-SE-positive cell was identified in

Figure 1 Histology of donor testes. In calves with SC between 18–20 cm, 45% of tubules contained a single layer of epithelium composed of spermatogonia (arrows) and Sertoli cells (arrowhead) (A), while the remaining tubules were composed of 2–4 layers with spermatocytes (arrows) (B). In calves with SC between 21 and 22 cm, nearly 53% of tubules contained 4–6 layers epithelium and spermatogenesis had progressed to production of spermatids (arrow) (C). Bar = 50 μ m.

Figure 2 Immunohistochemical identification of spermatogonia in bull calf seminiferous tubules using an antibody against PGP 9.5. In immature testis, immunoreactivity was strongly localized in the nuclear and faintly in the cytoplasm of gonocytes (arrow), while some of the gonocytes were not stained (arrowheads) (A). In prepubertal (SC 16 cm) (B) and pubertal testis (SC 22 cm) (C), spermatogonia (arrows) were positive for PGP 9.5, generally located on the tubular basal lamina and were round cells with a spherical nucleus. After enzymatic digestion of tubules to produce single cell suspensions, the percentage of cells positive for PGP 9.5 staining was calculated, excluding the positively stained cellular debris (arrows) (D). Bar = 50 μ m.

the recipient testes by the same examination procedures. High background fluorescence obscured any signal from CFDA-SE-stained cells.

Evaluation of transplant success using PKH26-dyed cells as markers

PKH26-labelled cells were identified in 15 out of the 25 testes (60%) injected with these cells. The period from transplant to castration was inversely related to the

detection of these PKH26-positive cells (Fig. 4). A higher percentage of testes from animals castrated within 10 weeks of transplant contained PKH26-positive cells than those animals castrated 20–30 weeks after transplant (5/5, 100% vs 10/20, 50%). In the five testes from animals castrated within 10 weeks of transplantation, a mean of 9% (range 4–18%) tubules contained PKH26-stained cells. This contrasted with a mean of 1% (range 0–3.6%) positive tubules when animals were castrated 20–30 weeks after transplant (Fig. 4).

Figure 3 PKH26-labelled donor germ cells in the seminiferous tubules of recipient bulls at different time points. A, B and C are samples from a testis castrated at 4 weeks following transplant. They illustrate positive results in intact recipient seminiferous tubules at low (A) and high magnification (C) together with a frozen section (B) showing a tubule outlined with positive cells. D, E and F illustrate PKH26-labelled cells in the seminiferous tubules of recipient testes, which were castrated at 10 weeks (D), 20 weeks (E) and 30 weeks (F) following injection. There was variation in the amount of fluorescence for individual cells which indicated either cell division or variable uptake of the stain during the staining procedure. A, Bar = 200 μ m; B–F, Bar = 50 μ m.

Figure 4 Percent positive tubules (a) and percent positive testes (b) from recipients castrated from 2 to 30 weeks after transfer of donor testis cells labelled with PKH26.

The maturity status of the donor testis (measured by SC at time of donor castration) was not a predictor of transplant success. Donor maturity (SC 18–20 vs 21–22 cm) did not affect the number of recipient testes with positive cells (7/10, 70% vs 8/15, 53% $P>0.05$) or the number of positive cells per testis (2.3 ± 0.7 vs 2.5 ± 1.1 $P>0.05$).

The maturity status of the recipient testis (measured by SC at the time of transplant: 15–18 vs 19–21 cm) did not affect the number of recipient testes with positive cells (7/11, 64% vs 8/14, 57%). Although there appeared to be more positive cells per testis in recipients with smaller SC at transfer (3.2 ± 1.2 vs 1.7 ± 0.6), this was confounded by the period from transplant to castration (Fig. 4a).

Long-term effect of transfer procedure on animals and testis

The calves were monitored for external signs of response to the transplanted cells for 3 days following the testis cell transfer procedure. No pain or swelling was observed in either testis and there was no increase in body temperature. After castration of recipients, there was no gross or histological sign of inflammation observed as a result of the transfer procedure. Testicular growth was assessed, according to monthly increase in scrotal size, following testis cell transfer. This was normal in 14/15 animals. SC increased by 1 cm every 23.2 days on average for the 15 recipients. However, in one of

these animals, the left testis was 28 g and the right was 114 g when removed at castration 164 days after testis cell transfer. The left testis was approximately the same size at castration as at the time of transfer.

Discussion

This work has demonstrated that male germ cell transplantation between unrelated bull calves of different breeds resulted in successful colonization of recipient seminiferous tubules by donor germ cells. This is the first and preliminary step towards confirming donor sperm production by recipient testes. Our result in cattle confirms previous work performed in goats and pigs (Honaramooz *et al.* 2002a, 2003a,b). Previously, germ cell transfer in cattle resulted in successful colonization only when autologous cells were used (Izadyar *et al.* 2003). Successful heterologous transplant in cattle raises the possibility of its use for commercial breeding purposes. For this to occur, long term donor sperm production by recipient testes will need to be documented.

We have demonstrated that PKH26 fluorescently labelled donor cells could be detected in 50% of recipient testes for up to 6 months. The percentage of PKH26-positive cells decreased over time such that very few were present after 6 months and detection frequency was maximal within the first month after injection. The objective in extending the evaluation period to 6 months was to observe the longevity of donor cell colonization, although examination at this late stage is likely to have resulted in false negatives as the fluorescence intensity of PKH26 in cells decreases during each division (Parish 1999). Overall, we interpret these results as suggesting that the transplanted donor cells will remain in the recipient testis for long enough to initiate spermatogenesis and thus the next phase of this experiment will be to identify donor sperm in the ejaculate of recipient animals via breeding trials or DNA comparison.

Our results contrast with the results of Izadyar *et al.* (2003), who showed that, while autologous transplantation resulted in colonization in 80% of recipients at 2.5 months after injection, no colonization was seen after homologous transplantation (Izadyar *et al.* 2003). There are three differences between the two studies that may account for the contrasting results. Izadyar *et al.* (2003) used highly enriched donor cell suspensions that were cryopreserved before transfer into older (10 month) recipients, whose testes had been treated with radiation. Additionally, no marker was used to identify the donor cells in that study, which may have limited its sensitivity in detecting donor cells in recipient tubules.

The immunological response to germ cell transplantation is of great interest, with contrasting results presented for allogeneic transplants in rodent and non-rodent species. Successful male germ cell transplantation in rodents requires transfer to syngeneic or

immunosuppressed recipients (Brinster & Zimmermann 1994, Dobrinski *et al.* 1999, 2000, Shinohara *et al.* 1999). In pigs and goats, allogeneic transfers have been successful (Honaramooz *et al.* 2002a, 2003b). In cattle, only autologous transplants resulted in colonization (Izadyar *et al.* 2003). However, in our study, colonization could occur in heterologous recipients of a different breed to the donor. We would expect that the donors and recipients would possess different histocompatibility antigens. Successful transfer across this transplantation barrier in cattle is in agreement with result obtained in pigs and goats (Honaramooz *et al.* 2002a, 2003b).

Despite introduction of 'foreign' cells into the testis of recipients, there does not appear to be any short- or long-term effect of the transplant procedure on testis function, although we have not evaluated this in detail. We did not observe obvious clinical impacts in recipients, as no external sign of inflammation was observed in recipient testes and the SC of recipient testes continued to expand as expected at approximately 1 cm/month. At castration, one recipient had one testis that remained the same size after transplant, while the other grew to an expected size of 114 g. The small testis could be the result of the transplant procedure or it is possible that at transplant, both testes were affected by a congenital germ cell deficiency (hypoplasia). If congenital hypoplasia was present at the time of testis cell transfer, the introduced stem cells may have 'rescued' the phenotype, and stem cell division was successful in only one testis, which then grew normally to 114 g at castration. This latter explanation is supported by the histological findings of a high proportion of Sertoli, the only tubules in the small (28 g) testis. The larger testis contained a small but significant number of Sertoli cell, the only tubules with the majority of tubules showing spermatogenesis. Nevertheless, further investigations into the effects of germ cell transplant on recipient testes are required to assess the impact of immunological factors on transplant success and to evaluate the animal welfare implications of the procedure.

In order to precisely assess persistence of colonization of recipient tubules, a donor cell marker is necessary. Transgenes have been used as markers in mice (β -galactosidase) (Brinster & Zimmermann 1994) and recently in goats (human α_1 antitrypsin) (Honaramooz *et al.* 2003b). As transgenic donor cells were not available in the present experiments, we used two different fluorescent dyes, PKH26 and CFDA-SE, to distinguish transplanted cells from endogenous spermatogenesis. Both the dyes have been used in immunological studies to track lymphocyte migration and proliferation for extended periods (Parish 1999). CFDA-SE is a comparable dye to PKH26 although CFDA-SE is easier to use and cheaper than PKH26. PKH26 has been used in germ cell transplantation experiments to label donor cells in goats for 1 month and in pigs for 3 months (Honaramooz *et al.* 2002a,b, 2003a). In our experiments, there was variation

in the amount of fluorescence in individual cells in the recipient testis, which indicated either cell division or variable uptake of the stain during the staining procedure.

The CFDA-SE fluorescent dye was not suitable for labelling testicular germ cells for transplantation due to the high background signal encountered when examining recipient testes. The fluorescent dye may be taken up by short lived molecules initially, then released and subsequently taken up by other cells during the first 24 h following labelling, creating high fluorescent background (Parish 1999). Dye concentration can affect cell viability and it is possible that the concentration of CFDA-SE used here reduced viability of transferred cells. It is also possible that CFDA-SE-stained cells were present in the recipient testes. However, we observed high background fluorescence in the testis cells, tubules and sections examined. Although this fluorescence was quenched within 10 s, we were unable to find positive cells with any certainty.

The pattern of donor cells observed in recipients was variable. Much of this variation appeared to be related to the period from transplantation to castration. Highest colonization rates were observed in recipients castrated within 1 month of transplantation. Within positive recipients it was common to observe patches of positive cells. This was most evident while examining tubule whole mounts (Fig. 3). These well colonized areas indicate that there are certain areas where the donor cells are most able to compete. This competitiveness may be due to random effects of these tubules filling with greater numbers of donor cells at the time of transplantation or perhaps due to vacant niches capable of accepting new stem cells.

The recipient animals in the present study were not depleted of endogenous stem cells. In other studies, removal of endogenous germ cells significantly increased donor stem cell colonization efficiency (Brinster & Zimmermann 1994, Shinohara *et al.* 2002). Although depletion of endogenous germ cells in recipient testes is not a prerequisite for successful spermatogonial cell transplantation (Shinohara *et al.* 2002), it will be one of the mechanisms to improve the success rate of transplantation procedure and thus these methods will be explored in further studies.

We did not observe an effect of donor testes tubule maturity on germ cell transplant outcome. We hypothesized that colonization rates would increase when cells from less mature testes were transplanted due to the higher percentage of spermatogonia present in cell suspensions following enzymatic isolation of tubule cells. We did find SC to be a good indication of testis maturity (tubular diameter, onset of meiosis). The maturity of recipient testes did not affect colonization rate. This is somewhat surprising as we envisaged that less mature recipients (presumably with a fewer layer of cells in seminiferous tubules) would be more likely to allow passage of donor stem cells to the basement membrane.

This study has demonstrated that germ cell transplantation between different bull calves, and indeed between cattle breeds, can be successful. However, this conclusion is preliminary as we have not documented donor sperm production in the recipient testis nor offspring derived from donor sperm. Thus, further studies will be needed to demonstrate the capacity of the donor cells to undergo spermatogenesis in recipient testes. Although the fluorescent dye, PKH26, was suitable for labelling donor testis cells for up to 6 months, it was not suitable for demonstrating spermatogenesis by the introduced donor cells. Subsequent research will investigate practical methods for stem cell depletion in recipients and the use of cultured stem cells to provide large numbers of cells for transplant. We envisage that a robust germ cell transplant technique for cattle could be developed by addition of these methods.

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References

- Aponte PM, de Rooij DG & Bastidas P 2005 Testicular development in Brahman bulls. *Theriogenology* **64** 1440–1455.
- Baker P & O'Shaughnessy P 2001 Role of gonadotrophins in regulating numbers of Leydig and Sertoli cells during foetal and postnatal development in mice. *Reproduction* **122** 227–234.
- Brinster RL & Zimmermann JW 1994 Spermatogenesis following male germ-cell transplantation. *PNAS* **91** 11298–11302.
- Dobriniski I, Avarbock MR & Brinster RL 1999 Transplantation of germ cells from rabbits and dogs into mouse testes. *Biology of Reproduction* **61** 1331–1339.
- Dobriniski I, Avarbock MR & Brinster RL 2000 Germ cell transplantation from large domestic animals into mouse testes. *Molecular Reproduction and Development* **57** 270–279.
- Hill JR & Dobriniski I 2006 Male germ cell transplantation in livestock. *Reproduction, Fertility and Development* **18** 13–18.
- Honaramooz A, Megee SO & Dobriniski I 2002a Germ cell transplantation in pig. *Biology of Reproduction* **66** 21–28.
- Honaramooz A, Snedaker A, Boiani M, Scholer H, Dobriniski I & Schlatt S 2002b Sperm from neonatal mammalian testes grafted in mice. *Nature* **418** 778–781.
- Honaramooz A, Behboodi E, Blash S, Megee SO & Dobriniski I 2003a Germ cell transplantation in goats. *Molecular Reproduction and Development* **64** 422–428.
- Honaramooz A, Behboodi E, Megee SO, Overton SA, Galantino-Homer H, Echelard Y & Dobriniski I 2003b Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. *Biology of Reproduction* **69** 1260–1264.
- Izadyar F, Den Ouden K, Stout TA, Stout J, Coret J, Lankveld DP, Spoormakers TJ, Colenbrander B, Oldenbroek JK, Van der Ploeg KD *et al.* 2003 Autologous and homologous transplantation of bovine spermatogonial stem cells. *Reproduction* **126** 765–774.
- Jiang FX & Short RV 1995 Male germ cell transplantation in rats: apparent synchronization of spermatogenesis between host and donor seminiferous epithelia. *International Journal of Andrology* **18** 326–330.
- Joerg H, Janett F, Schlatt S, Mueller S, Graphodatskaya D, Suwattana D, Asai M & Stranzinger G 2003 Germ cell transplantation in an azoospermic klinefelter bull. *Biology of Reproduction* **69** 1940–1944.
- Kanatsu-Shinohara M, Ogonuki N, Inoue K, Ogura A, Toyokuni S, Honjo T & Shinohara T 2003 Allogeneic offspring produced by male germ line stem cell transplantation into infertile mouse testis. *Biology of Reproduction* **68** 167–173.
- Lunstra DD, Ford RR & Echternkamp SE 1978 Puberty in beef bulls: hormone concentrations, growth, testicular development, sperm production and sexual aggressiveness in bulls of different breeds. *Journal of Animal Science* **46** 1054–1062.
- Parish CR 1999 Fluorescent dyes for lymphocyte migration and proliferation studies. *Immunology and Cell Biology* **77** 499–508.
- Rathi R, Honaramooz A, Zeng W, Schlatt S & Dobriniski I 2005 Germ cell fate and seminiferous tubule development in bovine testis xenografts. *Reproduction* **130** 923–929.
- Russell LD, Ettlin RA, Sinha-Hikim AP & Clegg ED 1990 *Histological and Histopathological Evaluation of the Testis*, 1st edn Clearwater: Cache River Press, p 286.
- Shinohara T, Avarbock MR & Brinster RL 1999 beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. *PNAS* **96** 5504–5509.
- Shinohara T, Orwig KE, Avarbock MR & Brinster RL 2002 Germ line stem cell competition in postnatal mouse testes. *Biology of Reproduction* **66** 1491–1497.
- Wrobel KH 2000 Prespermatogenesis and spermatogoniogenesis in the bovine testis. *Anatomy and Embryology* **202** 209–222.
- Wrobel KH, Bickel D, Kujat R & Schimmel M 1995 Configuration and distribution of bovine spermatogonia. *Cell and Tissue Research* **279** 277–289.
- Zhang Z, Renfree MB & Short RV 2003 Successful intra- and inter-specific male germ cell transplantation in the rat. *Biology of Reproduction* **68** 961–967.

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