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Long-term follow-up of porcine male germ cells transplanted into mouse testes

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Summary

This study investigated the effect of increased phylogenetic distance on the outcome of spermatogonial transplantation, with porcine donors and mice recipients. It was designed to develop a technique for detecting foreign donor cells in recipient animals. Porcine male germ cells were harvested from postnatal male testes and incubated with the lipophilic membrane dye PKH-26. For transplantation, approximately 10^6 PKH-26-labelled porcine male germ cells were injected into the efferent ducts of mouse testes. Animals were sacrificed at post-graft days 1, 10, 30, 45, 60 and 150 ($n = 5$ each). Serial frozen sections of explanted testes were prepared for detecting labelled cells. Transplanted porcine donor cells were easily detected in the recipient tubules for 8 weeks. After transplantation, we could detect both incorporation into the basement membrane and differentiation of grafted porcine donor cells by our double detection system, using PKH staining and slide PCR. However, our RT-PCR and apoptosis results revealed that most of the grafted porcine male donor cells could not differentiate past early-meiotic spermatocytes. We could induce partial differentiation of xenografted porcine donor cells in mouse testes, but not full induction of spermatogenesis. We have developed a very reliable technique for detecting foreign donor cells in recipient animals using a combination of PKH staining and slide PCR methods. Our results provide a valuable experimental model for applying and evaluating this technology in other species.

Keywords: PKH-26 stain, Slide PCR, Xenotransplantation

Introduction

In Western countries, one out of seven couples experiences fertility problems, of which at least half are of unknown paternal etiology (Olive & Cuzin, 2005). Therefore, application of germ-cell transplantation technology in mammals as a treatment tool may be more practical than conventional reproductive technologies (Orwig & Schlatt, 2005).

Approximately 95% of the cells in normal adult testes are differentiating germ cells (Kubota *et al.*, 2003). Treatment with an appropriate cytotoxic dose of the alkylating agent, busulfan, depletes most spermatogenic cells in the testis. However, testicular somatic cells, including Sertoli and Leydig cells, are unaffected (Kim *et al.*, 1997; Choi *et al.*, 2004, 2006). Recently, our group reported that the depletion of male germ cells in busulfan-treated mice is mediated by a loss of c-*Kit*/SCF signalling, but not by p53-dependent or Fas/FasL-dependent mechanisms (Choi *et al.*, 2004). Accordingly, treatment of male mice with busulfan is employed to obliterate endogenous germ cells to prepare recipients for germ-cell transplantation from a donor male (Brinster & Avarbock, 1994).

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Although the low success rate for germ-cell transplantation into recipient testes limits the practical application of this technique to experimental use, transplantation of xenologous male germ cells into animal testes has been extensively explored in rodents (Oatley *et al.*, 2002). The technique is likely to find its most valuable and practical applications in other species (Schlatt *et al.*, 1999). For example, the spermatogonial transplantation system has obvious clinical applications for restoring spermatogenesis in men or boys whose fertility has been compromised by irradiation or chemotherapeutic treatment for cancer.

Interspecies spermatogonial transplantation provides a unique system for studying the cellular and molecular events that regulate the sequential steps of spermatogenesis (Ohta & Wakayama, 2004). Increased knowledge of the factors controlling spermatogonial proliferation and differentiation should aid in understanding disruptions in spermatogenesis and enable manipulation of the microenvironment of recipient seminiferous tubules to support spermatogenesis of phylogenetically distant donor species. The spermatogenesis of rat germ cells transplanted into mouse testes were supported by mouse Sertoli cells, with only occasional minor defects (Russell & Brinster, 1996). Transplantation of hamster male germ cells into mouse seminiferous tubules resulted in the production of hamster sperm (Ogawa *et al.*, 1999). However, morphological abnormalities in spermatogenesis were frequently detected. Furthermore, germ cells from rabbits and dogs colonized mouse seminiferous tubules, but did not differentiate beyond the stage of spermatogonial expansion (Dobrinski *et al.*, 1999), indicating that the increased phylogenetic distance between the donor and recipient species results in decreased efficiency of transplanted germ-cell spermatogenesis. Although the phylogenetic distance between rabbits and mice is approximately equal to that between dogs and mice (Brinster & Avarbock, 1994; Johnston *et al.*, 2000), stem cells from rabbits are more successful in colonizing recipient testes. This observation indicates that the potential success of transplanted germ-cell spermatogenesis is not predictable solely by phylogenetic distance, but also involves species-specific factors, which can be studied experimentally (Brinster *et al.*, 2003). Therefore in the present study, we address the effect of the increased phylogenetic distance between porcine donors and mice recipients on the outcome of spermatogonial transplantation. Moreover, xenogenic transplantation to animal recipient testes provides valuable information about male germ-line stem cells. Full implementation of testis cell transplantation techniques in other species will require protocols that distinguish donor-derived male germ cells. Thus, an effective bioassay is necessary to distinguish progeny cells derived from donor male stem cells and endogenous male germ cells developing in recipient testes. In the present study we used both PKH staining and slide PCR to distinguish between grafted and endogenous male germ cells. In particular, we examined whether porcine male donor cells are successfully settled and repopulated in mouse seminiferous tubules and use a combination of PKH staining and slide PCR as a tool for detecting the foreign donor cells in the absence of specific antibodies.

Materials and methods

Animals and treatment

Mice (30–40 g, 8–12 weeks old) were housed in cages and fed *ad libitum*. Mice and pig maintenance and experiments were conducted in accordance with the Kon-Kuk University guide for the *Care and Use of Laboratory Animals*. Eight-week-old ICR male mice received a single intraperitoneal injection of busulfan (40 mg/kg body weight) diluted in sesame oil, as described previously (Kim *et al.*, 1997). Of these mice, 18 were used as placebo controls following busulfan treatment and 30 were used for the transplantation of porcine male germ cells. Of the latter, 14 mice testes were paraffin-embedded for slide PCR or frozen-sectioned for confirming PKH-26 staining of porcine male germ cells and 16 mice testes were used for flow cytometry and RT-PCR.

Preparation of porcine male germ cells

Porcine male germ cells (Landrace × Duroc F1) were prepared according to previously described methods with minor modifications (Brinster & Avarbock, 1994). The testes from four out of 12 5–7-day-old pigs were used for histological analysis and/or flow cytometry analysis after PKH-26 staining. Testes from the remaining eight pigs were used for mouse testes transplantation. Briefly, the porcine testes were decapsulated to remove the tunica albuginea and minced. The dissociated tissue was incubated in DMEM/F12 (Gibco) containing collagenase type I (1 mg/ml), hyaluronidase (0.5 mg/ml) and DNase I (5 μg/ml) for 15 min at 34°C with gentle agitation. All enzymes were purchased from Sigma. Laminin-coated plates (60 mm) were pre-incubated for 30 min at 34°C and 4×10^7 single cells were suspended in 8 ml DMEM/F12 containing 10% fetal bovine serum (FBS; HyClone). Cells were incubated for 30 min at 37°C, followed by two washes with PBS to remove unbound cells. Attached cells were removed by trypsin (0.25%)–EDTA (1 mM) digestion for 3 min at 37°C, followed by strong pipetting and filtered through a 60 μm nylon mesh (Millipore) to remove undigested clumps.

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Fluorescence labelling

Porcine male germ cells were transferred to polystyrene tubes and washed twice with serum-free medium. The cells were resuspended in loading buffer (an aqueous osmolarity-regulating solution containing no Ca^{2+} or other physiological salts; Sigma). PKH-26 (0.5 µl of 1 mM stock in ethanol; Sigma) in 100 µl buffer was used for loading. The dye solution was added slowly while agitating the cell suspension on a shaker to avoid over-staining. After 30–40 min of shaking at RT, the staining reaction was terminated by incubation with 500 µl FBS for 30 s at RT. After centrifugation, the cell pellets were transferred into fresh 50 ml tubes (Falcon, France) and washed twice with 40 ml of RPMI containing 10% FBS. After the last wash, the cells were resuspended at a final concentration of 10^6 cells/µl. Trypan blue was added to the cell suspensions to monitor the successful transplantation of the porcine male germ cells into mouse seminiferous tubules.

FACS analysis and compensation

FACS analysis was performed with a FACScan Cytometer (Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA). Using an excitation wavelength of 488 nm, the fluorescence of the three fluorochromes was recorded at 530 nm for FITC (FL1), 585 nm for PKH-26 (FL2) and >675 nm for PI (FL3). Germ cell viability was analyzed with a viability assay kit (LIVE/DEAD® Viability/Cytotoxicity Assay Kit, Molecular Probes) by using flow cytometry.

Transplantation of porcine germ cells into mouse testes

Spermatogonial enrichment and germ-cell transfer were performed according to our previously reported method (Choi *et al.*, 2006). Mice were anesthetized and placed in dorsal recumbence. The testes were exteriorized and viewed by stereomicroscopy (Olympus SZX12). The solution (10 µl) containing 6×10^6 donor cells were injected into the efferent ducts under stereomicroscopy. The successful transplantation of porcine male germ cells into the seminiferous tubules of 47 of the 60 testes from 30 mice was confirmed by the presence of trypan blue. The failed testes were removed by surgical methods.

Slide PCR

For paraffin embedding, fresh tissues were fixed in 10% formalin at RT for up to 24 h and dehydrated twice for 30 min in 70% ethanol, followed by 30 min in 100% ethanol. Following infiltration of the tissues with paraffin, they were embedded into paraffin blocks for storage until microtome sectioning. Sections (4 µm) were cut on poly-L-lysine-coated glass slides. Tissue sections were dewaxed with xylene for 30 min and 100% ethanol for 5 min. Samples were gradually rehydrated using a graded alcohol series and digested with 20 µg/ml proteinase K (Boehringer Mannheim) in PBS for 10 min at 37°C. The samples were washed extensively in PBS to inactivate the enzyme. Slides were dehydrated in a graded alcohol series and amplification was performed using *in situ* thermal cycling (MJ Research, Waltham, MA, USA). The PCR reaction for porcine germ cell detection in transplanted mouse testes was performed using porcine uroplakin II (upk2, AY044189) forward and reverse primers: 5′-CCGCTCAGAAGCTGGGCTGGTGCT-3′ and 5′-GGTACCCAGCATCAATTGGCT-3′, respectively (Kwon *et al.*, 2002). PCR reaction mixtures (65 µl) contained 1 µl of *Taq* polymerase (AT gene), 6 µl of 10× PCR buffer, 6.5 µl of 10× DIG DNA Labelling Mix (Roche), 1 µl of each primer (10 nM) and 49 µl of autoclaved H_2O. Slides were warmed to 94°C for 5 min to perform hot-start PCR and 65 µl of the PCR reaction mixture was placed directly on the tissue section, covered with slide seals (TaKaRa Biomedicals, Japan) and cycled as follows: 94°C for 1 min, 58°C for 1 min and 72°C for 1 min (16 cycles). Slide seals were removed and the tissues were washed in PBS for 5 min. Samples were incubated for 30 min with anti-digoxigenin AP (1:500 dilution) prepared in blocking buffer. Slides were washed three times in Tris buffer solution (pH 7.6). Freshly prepared 5′-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT; Sigma) was used as the chromogenic substrate. Slides were covered with the substrate and monitored for colour development.

TUNEL assay (TdT-mediated dUTP-X nick end labelling)

Testes were fixed in 4% (w/v) paraformaldehyde in 0.01 M D-PBS (Gibco, Dulbecco's phosphate-buffered saline (PBS)), washed in PBS, dehydrated in ethanol (70, 90 and 100%) and embedded in paraffin wax. Testicular sections (5 µm) were rehydrated (xylene 5 min; ethanol 100%, 95%, 70%, 2 min each) and washed in distilled water prior to TUNEL staining. The sections were incubated for 15 min with proteinase K (20 µg/ml) at room temperature and washed with PBS (1×) and endogenous peroxidase activity was blocked with 2% H_2O_2 for 5 min. Sections were re-washed three times with PBS (1×) and incubated for 60 min at 37°C in a moist chamber with the TUNEL mix (0.3 U/µl calf thymus terminal deoxynucleotidyl transferase, 7 pmol/µl biotin dUTP, 1 mM cobalt chloride in 1× reaction buffer in distilled water). After washing (four PBS baths of 5 min each at RT), the sections were saturated in 2% BSA for 10 min at RT. Sections were treated for 30 min at 37°C in a moist chamber with a

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1:20 dilution of ExtraAvidin peroxidase antibody. After three PBS washes, detection was performed with DAB [1.24 mg DAB, 25 µl 3% $NiCl_2$, 152 µl 1 M Tris–HCl (pH 7.5) in 2 ml distilled water]. Slides were mounted in crystal mount (Biomeda).

RT-PCR analysis

Tissue samples were obtained from each testis after transplantation. Samples were snap-frozen in liquid nitrogen and stored at –80°C until analysis. For the first-strand cDNA synthesis, a reverse transcription (RT) reaction was performed on 2 µg of total RNA prepared from each testis in a final volume 20 µl. The RT mix contained 1× RT buffer, 10 mM dithiothreitol (DTT), 0.5 mM each dNTP 0.5 µg Oligo dT, 10 U RNasin ribonuclease inhibitor and 200 U superscript reverse transcriptase. The RT reaction was carried out at 42°C for 50 min, followed by heating at 70°C for 15 min to inactivate the reaction. PCR analyses were performed in a final volume of 50 µl containing the cDNA sample, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCI (pH 8.3), 1.2 mM of each dNTP, 0.4 µM of each primer and 2 U *Taq* polymerase (ATgene). The amplifications of c-*kit* and protamine 1 (prm1) partial cDNAs were performed with specific primers: c-*kit* (AJ223231), forward 5′-CTAGACCTGGAGGACTTGCT-3′ and reverse 5′-CGCACGATCTGCTTAAACGT-3′; prm1 (NM 214253), forward 5′-TCTGAGCATCAAGACTGAGT-3′ and reverse 5′-TTGTGCTTAGCAGGCACCTGTCA-3′.

Statistical analysis

All experimental data are presented as means ± SDs. Each experiment was performed at least three times and subjected to statistical analysis. Representative experiments are presented. For statistical analyses, one-way analysis of variance (ANOVA) was performed to determine the differences between the groups. The Fisher's post-test was performed to determine the significance between pairs of groups. A *P*-value < 0.05 was considered significant. Statistical tests were performed with StatView software version 5.0 (SAS Institute Inc.).

Results

Preparation of recipient mouse and donor porcine testes

Initially, we investigated the effects of busulfan on whole testes and developing male germ cells in sections of mouse testes. Our previous study showed that testicular weight dropped in busulfan-treated mice, commencing after 1 week and reaching its lowest point 4 weeks after busulfan injection (33% of the control value) (Choi *et al.*, 2004). Testicular weight progressively recovered thereafter, reaching approximately 90% of the control weight at 12 weeks (Choi *et al.*, 2004). Four weeks after the injection of a single dose of busulfan, male germ cells were significantly reduced in number and most of the spermatogenic cells, except spermatogonia, were absent (Fig. 1*B*) as compared to the control mice (Fig. 1*A*).

Initially, we checked the viability of extracted porcine testes from 8-day-postnatal animals by electron microscopic morphometry (Fig. 1*C*) and haematoxylin and eosin staining (Fig. 1*D*). The extracted testes were normal and we could not detect any differentiating or differentiated cells.

In vitro analysis of stability and viability of PKH-26-labelled porcine male germ cells

Recently we reported that staining with an optimal concentration of PKH-26 (2 µM) resulted in stable loading with high fluorescence intensity in spermatogonia (Choi *et al.*, 2006). In contrast, over-staining of spermatogonia with 10 µM PKH-26 led to the accumulation of intensively stained single spermatogonia, resulting in artificial light emission (Choi *et al.*, 2006). This potentially interferes with the detection of double-positive target cells and leads to false positive results in the cytotoxic assay. The 2 µM concentration used in this experiment provides complete staining without over-stained spermatogonia. Notably, putative porcine spermatogonial stem cells showed high intensity staining with PKH-26 upon loading (Fig. 1*E*, *F*). The PKH-26 label remained stable in all vital cells tested for a minimum of 72 h with no loss of fluorescence intensity. Next, we examined whether staining with PKH-26 altered spermatogonia viability (Fig 1*G*, *H*). A dead and live stain assay performed before (Fig. 1*H*, control) and 24 h after (Fig. 1*H*, culture) loading with PKH-26 revealed no significant differences.

Fate of porcine male germ cells transplanted into mouse testes

To determine whether porcine male germ cells could be incorporated into mouse testes and produce mature sperm cells *in vivo*, PKH-labelled porcine male germ cells were injected into the mouse efferent duct 4 weeks after busulfan treatment and the incorporation was determined by trypan blue injection (Fig. 2). Host testes were removed 1–60 days post-transplantation and viewed in whole-mount preparations to assess the distribution of donor cells along the seminiferous tubules. At 1 day post-transplantation, PKH-26-labelled porcine donor cells were identified as distinct clusters along the seminiferous tubules (Fig. 3*B1*, *B2*). We also examined cryostat sections taken from

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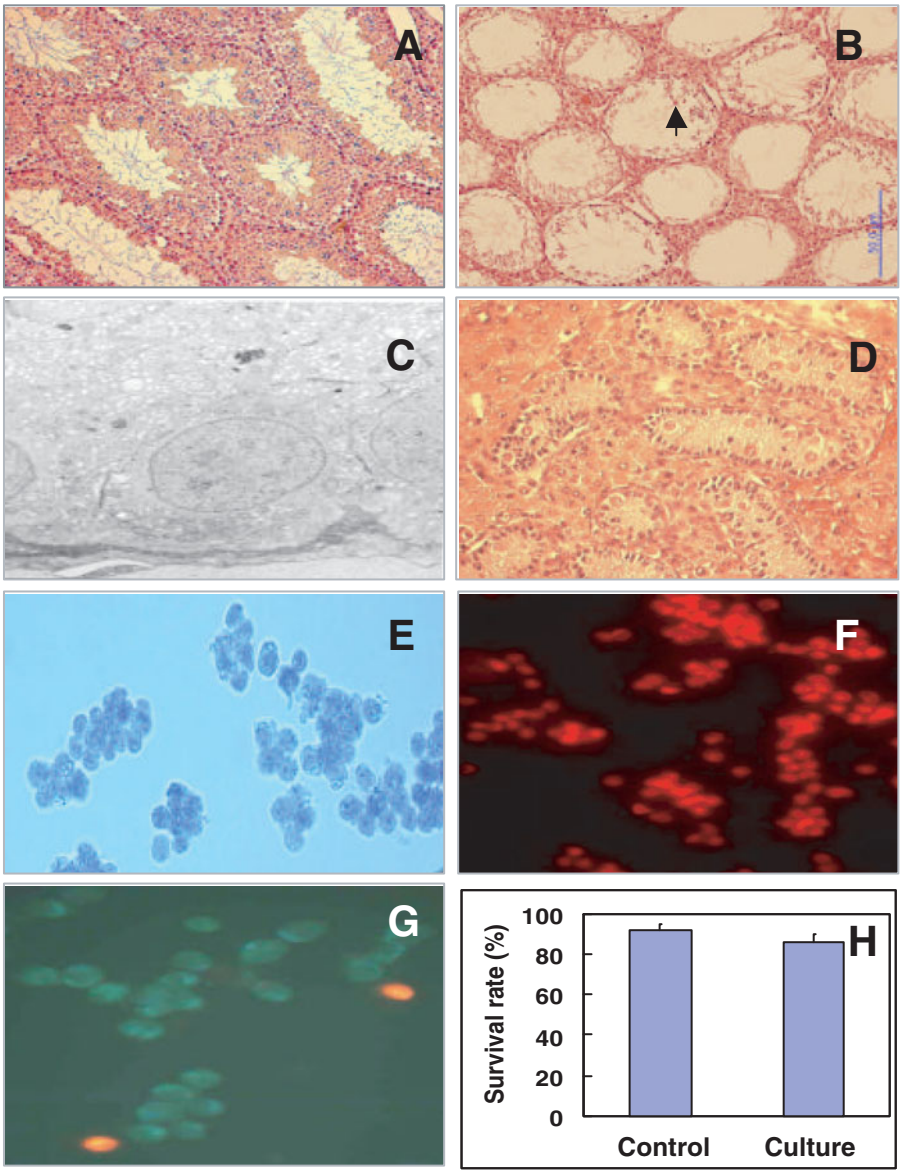

Figure 1 Photomicrographs of donors and recipients. Testes at 4 weeks after busulfan treatment (*B*), compared with those of control mice (*A*). Four weeks after busulfan treatment, the testes of most busulfan-treated mice are depleted of developing germ cells, although they contain somatic cells and spermatogonia. Arrow in (*B*) indicates debris from differentiating male germ cells. Porcine testes assessed by electron microscopy (*C*) and haematoxylin and eosin staining (*D*), indicating that most spermatogonial stem cells survived. Porcine male germ cells were obtained from 7–15-day-neonate testes (*E*) and stained with PKH-26 dye (*F*). The viability of porcine germ cells was examined using a live and dead staining kit. Green fluorescence represents live porcine germ cells and red fluorescence signifies dead porcine germ cells (*G*). Apoptotic cells in busulfan-treated testes were detected by flow cytometry analysis (*H*).

testes at 1 day post-transplantation. At this early time point, most porcine donor cells were identified in the centre of seminiferous tubules close to the lumen (Fig. 3*B3*, *B4*). At 10 days post-transplantation, a large number of porcine male germ cells were identified along seminiferous tubules in the vicinity of the injected efferent tracts (Fig. 3*C1*, *C2*) and most donor cells were localized in the basal layer of the seminiferous tubule (Fig. 3*C3*, *C4*). Examination of whole-mount preparations revealed that cells were more widely distributed across the seminiferous tubules than at 1 day post-transplantation. At 30 days post-transplantation, grafted donor cells survived along the seminiferous tubules (Fig. 3*D1*, *D2*) and were differentiated to early meiotic stages, extending numerous fine processes in the seminiferous tubules (Fig. 3*D3*, *D4*). At 60 days post-transplantation, although the transplanted cells adhered to the

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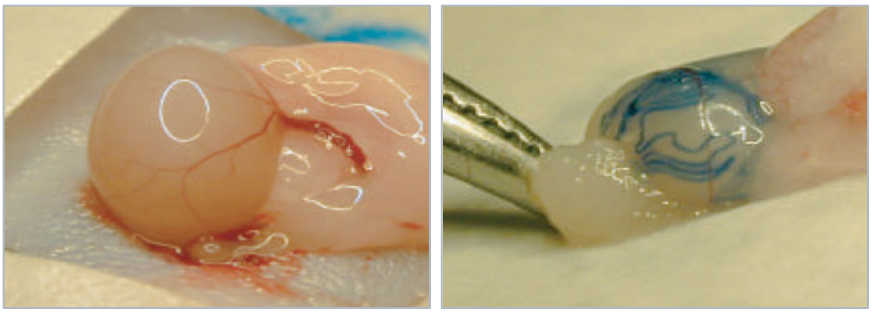

Figure 2 Microinjection of porcine donor cells into mouse testis. To confirm a successful transplantation, trypan blue was added to the injection solution. The tip of the micropipette was inserted into the testis and the head of the epididymis. Following a pressure increase in the pipette, the cell suspension was transferred to seminiferous tubules (dark blue).

Table 1 Development of porcine germ cells in mouse recipient testes

Time of analysis (days)	No. of recipients injected	No. of live recipients	No. of testes injected	No. of testes with spermatogenesis (%)	No. of tubules with spermatogenesis (%)
1	3	3	3	3/3 (100)	40/83 (48)
10	4	4	5	4/5 (80)	37/194 (19)
30	4	4	6	4/5 (80)	33/97 (34)
60	3	3	3	3/3 (100)	5/84 (6)

basement membrane of seminiferous tubules, donor cell-derived differentiated germ cells were rarely observed (Fig. 3*E3*, *E4*). Multiple nuclei, indicative of fusion with host cells, were not observed in the transplanted cells. Furthermore, we found no evidence of uncontrolled replication of porcine male germ cells after transplantation. This suggests that far longer follow-up times are possible with PKH-26 as an *in vivo* marker for cells that do not divide, since dividing cells share the stained surface of their parent cells, markedly decreasing the brightness.

To estimate the percentage of donor cells and donor cell-derived progeny cells, we performed slide PCR using porcine-specific primers for upk2 (Fig. 4, Table 1). In accordance with the PKH staining data, slide PCR revealed the presence of differentiated porcine male germ cells in 30-day post-transplantation samples (Fig. 4*D*), suggesting that the combination of PKH staining and slide PCR is a very reliable tool for the detection of foreign donor cells.

To determine whether porcine spermatogenesis actually occurred in recipient mouse testes, we analyzed the mRNA levels of porcine c-*kit* and *prm1* as pre-meiotic and post-meiotic germ-cell markers, respectively. RT-PCR using porcine c-*kit*- and *prm1*-specific primers revealed expression in porcine spermatogonia and early spermatocytes (Fig. 5). Furthermore, porcine *prm1* expression, but not c-*kit* expression, was detected for at least 45 days after transplantation (Fig. 4*E*), indicating that grafted porcine donor cells differentiated primarily into spermatids (Fig. 4*E*). These data suggest that transplanted donor cells efficiently produce spermatocytes but do not develop into mature sperm cells.

To address the relationship between donor-cell differentiation and cell survival rates, we examined the programmed cell death of donor cells and cell-derived progeny. Apoptotic cells at 4 weeks following placebo injections were detected as Leydig cells in recipient mouse testes (Fig. 6*A*, arrows), whereas marked apoptosis was observed in germ-cell-transplanted testes (Fig. 6*B*, arrows). These data indicate that although some porcine spermatogonia differentiate into post-meiotic spermatid in mouse testes, most of the grafted porcine donor cells do not fully differentiate past early-meiotic spermatocytes due to apoptosis.

Discussion

In this study, we show that transplanted porcine male stem cells efficiently produce spermatocytes that differentiate into haploid cells *in vivo*, but fail to develop into mature sperm cells. We employed adult ICR mice as recipient animals, following busulfan treatment to reduce the endogenous germ-cell numbers. The resulting seminiferous tubules contained only spermatogonia and Sertoli cells in a single cell layer. Therefore, donor cells could easily reach the basement membrane, without the migration through multiple layers of spermatogenic cells, as a first step

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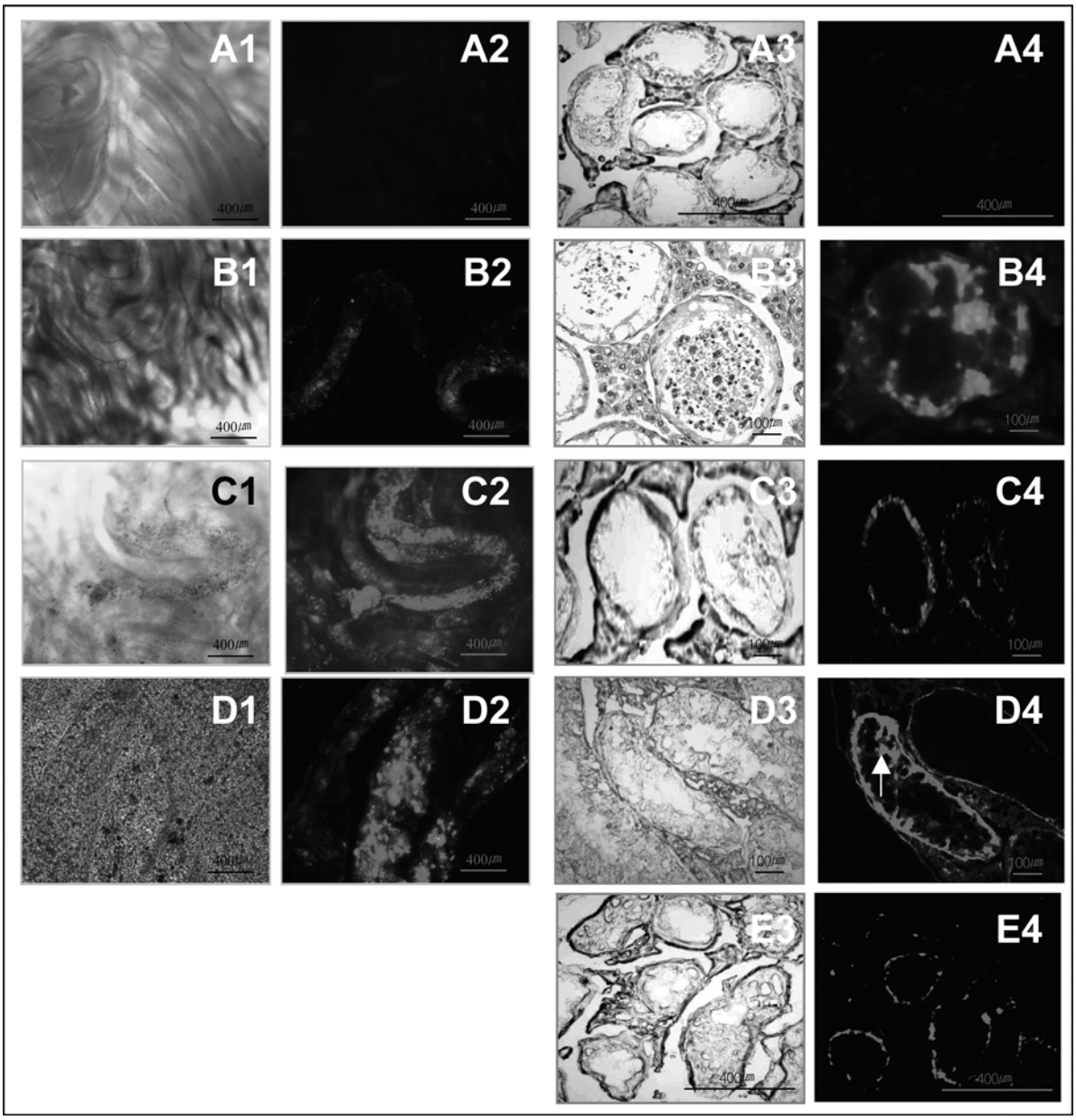

Figure 3 Xenotransplantation of porcine germ cells into germ-cell-depleted mouse testes. Photomicrographs of dispersed seminiferous tubules (*A1–D1*) and fluorescent-labelled (PKH-26) cells at 1 day (*B1*, *B2*), 10 days (*C1*, *C2*) and 1 month (*D1*, *D2*) after xenotransplantation of porcine germ cells. *A1* and *A2* are non-injected control seminiferous tubules. Cross-sections of recipient mouse testes after transplantation of porcine male germ cells assessed by light microscopy (*A3–E3*) and fluorescence microscopy (*A4–E4*) after 1 day (*B3*, *B4*), 10 days (*C3*, *C4*), 1 month (*D3*, *D4*) and 2 months (*E3*, *E4*). *A3* and *A4* are non-injected controls. At 1 day after transplantation, most porcine donor cells are located at the centre of the seminiferous tubules (*B4*) and translocate to the basement membrane of the seminiferous tubules at 10 days after xenografting (*C4*). Donor cells from some seminiferous tubules are differentiated into spermatocytes), but most donor cells are arrested at the spermatogonia stage (*E4*). The arrow in *D4* indicates that some porcine spermatogonia are differentiating into early meiotic stages. Figures are in ×200 magnification, except *A3*, *A4*, *E3* and *E4* (×100).

toward successful colonization. Testes at 1–8 weeks after germ-cell transplantation displayed significant PKH deposits, whereas control testes were rarely stained. In this mouse model of developing germ-cell-depleted testes, grafted porcine donor cells survived at least 150 days following transplantation and were associated closely with, or were incorporated into, the testis without significantly affecting seminiferous tubule architecture. The cells differentiated primarily into spermatids *in vivo*.

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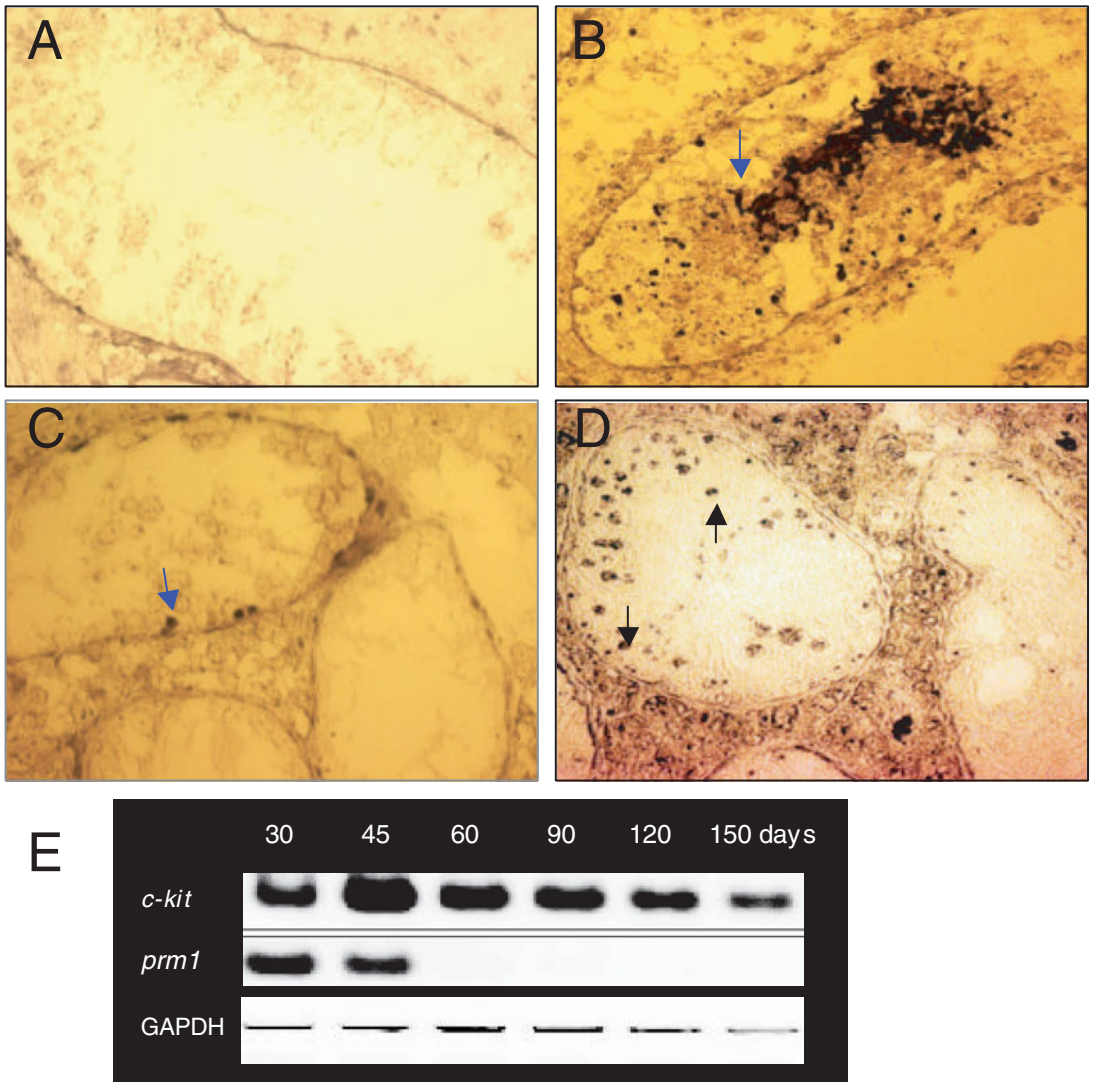

Figure 4 Detection of porcine germ cells in recipient mouse testes by slide PCR. Evaluation of gene expression in cryosections of recipient testes: control (*A*) and 1 day (*B*), 10 days (*C*) and 1 month (*D*) after xenotransplantation of donor cells. Porcine gene-expressing cells are located towards the periphery of seminiferous tubules. The arrows in *B* and *C* indicate porcine male germ cells near the lumen of seminiferous tubules shortly after transplantation and porcine-derived spermatogonia settled in the mouse testes, respectively. The arrows in *D* indicate that some porcine-derived male germ cells have differentiated into spermatocytes. (*E*) Analysis of donor cell-specific marker gene expression. Porcine c-*kit* expression patterns were relatively constant, whereas *protamine* (*prm1*) expression was not observed at 2 months after germ-cell transplantation. GAPDH, glyceraldehyde phosphate dehydrogenase.

PKH-26 is a lipophilic dye developed by Horan & Slezak (Horan & Slezak, 1989). It is the longest-lasting member of a family of cell-surface markers used for multiple purposes, such as *in vivo* tracking of various types of cells. No side effects on cell adhesion or proliferation are evident (Horan *et al.*, 1990). In the user manual, the supplier warranties a half-life of elution from rabbit red blood cells of 'greater than 100 days *in vivo*' (Sigma, 1993). Previous studies have indicated that maturation from porcine spermatogonia to mature sperm cells *in vivo* takes about 60 days (Franca & Cardoso, 1998). Accordingly, our study design was based on a maximum follow-up of 60 days, which seemed a reasonable period for producing mature sperm cells from porcine male stem cells. The possibility remained that PKH brightness might decrease markedly, since dividing cells would share the stained surface of the parent cells. To estimate the number of donor cells and donor-cell-derived progeny cells, we performed slide PCR using porcine-specific primers for *upk2*. In contrast to the PKH-26 data, slide PCR revealed the presence of differentiated porcine male germ cells (Fig. 4), suggesting that a combination of PKH staining and slide PCR is a very reliable tool

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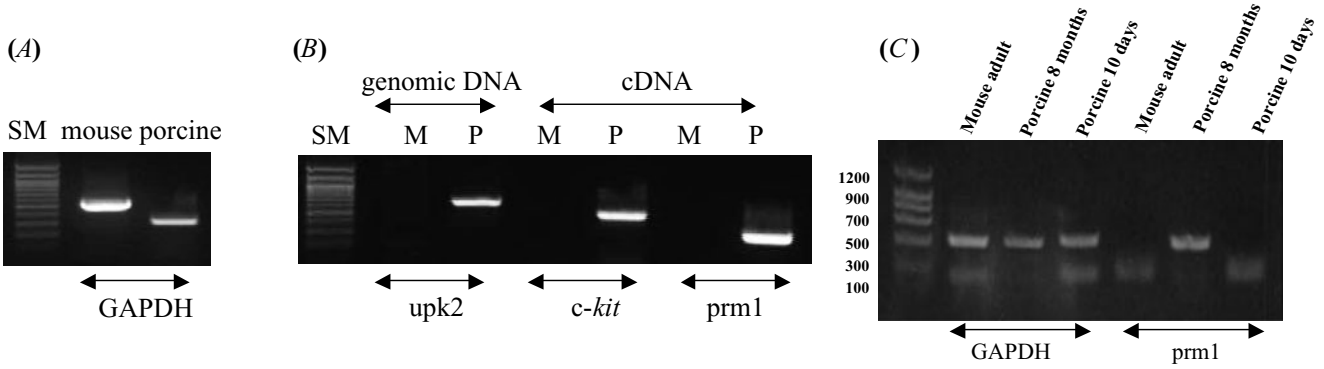

Figure 5 PCR primers in this study are porcine specific. (*A*,*B*) primer sequences and PCR conditions are described in the Materials and methods section. (*C*) Evidence for differentiation into haploid cells due to prm1 expression. GAPDH was detected both in mouse and porcine. The porcine adult testes is a positive marker for prm1. SM, size marker; M, mouse testis cDNA; P, porcine testis cDNA.

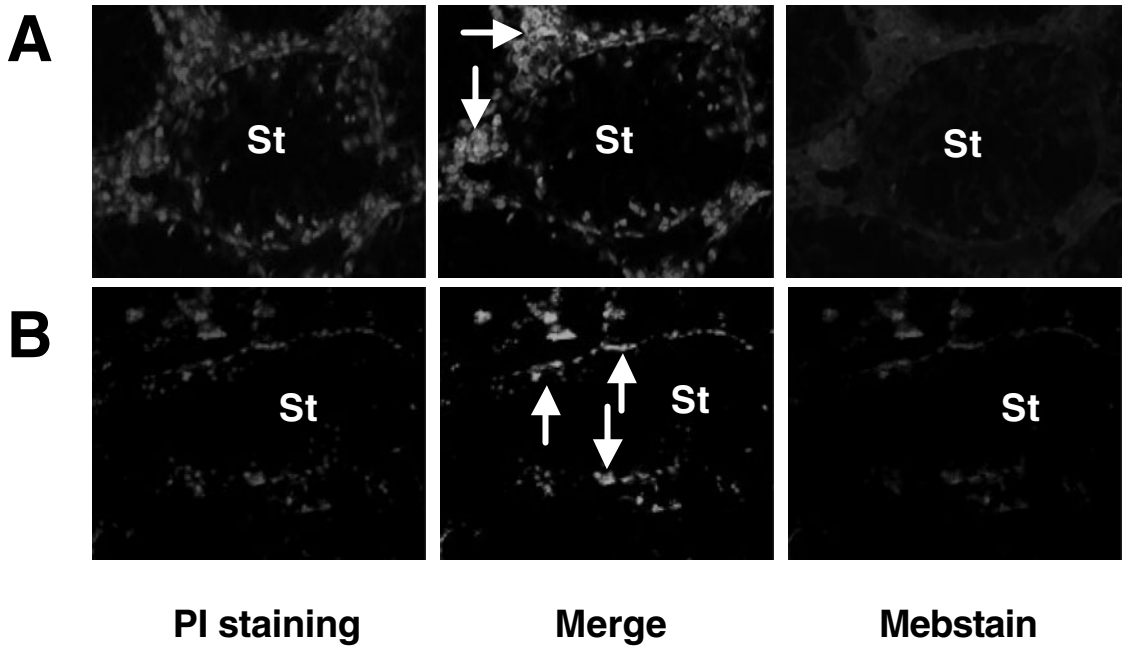

Figure 6 Monitoring of donor-derived male germ cell apoptosis by the TUNEL assays. For assay details, see Material and methods. Male germ cell apoptosis is indicated by brown colouration or green colour. Apoptotic cells in busulfan-treated control testes (*A*) are mainly limited to Leydig cells, whereas xenografted testes (*B*) include spermatogonia and/or meiotic germ cells, indicating that some pig male germ cells differentiated into spermatocytes despite extensive apoptosis in the recipient testes.

for the detection of foreign donor cells in the absence of specific antibodies.

Efferent duct transplantations induce widespread distribution of donor stem cells across seminiferous tubules. Mouse Sertoli cells have been shown to the support extensive migration and long-term survival of transplanted porcine donor cells, but the donor cells showed no further maturation (Dobrinski *et al.*, 2000). In the present study, transplanted cells were widely dispersed across the basement membrane of seminiferous tubules, particularly at longer post-transplantation times, with a tendency to spread out and cover as large an area of the seminiferous tubules as possible (Figs 2, 3). However, global distribution of donor cells across the seminiferous tubules was not always uniform. For instance, some areas of the seminiferous tubules did not contain any transplanted germ cells on the basement layer or within the tubules, whereas large patches of donor cells were present in other areas of the same seminiferous tubules. These data suggest that unequal distribution of grafted donor cells across seminiferous tubules is caused by

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differences in barrier integrity for the migration of grafted donor cells from the lumen to the basement layer.

The major finding of this study is the formation of porcine spermatocytes in mouse testes, suggesting that the ICR mouse testis does not completely prevent porcine spermatogenesis. For these transplantations, donor male germ cells were isolated from day-5 or day-7 postnatal porcine testes and the germ cells were selected on laminin dishes. Therefore, the donor germ cells may be pro-spermatogonia or authentic spermatogonia stem cells. Despite extensive apoptosis in the recipient testes, some male stem cells differentiated into spermatocytes (Fig. 2*D4*, *E4*). This observation is of interest because a previous report did not find any further maturation in the mouse testis (Dobrinski *et al.*, 2000). This result may illustrate the difficulty of relying on individual markers to identify donor cells. It is also possible that this discrepancy may be due to the recipient strain or the donor-cell detection methods used. In our study, we estimated the presence of donor cells and donor cell-derived progeny cells using PKH-26 staining and slide PCR. Collectively, our data illustrate the effectiveness of these methods for the identification of transplanted donor cells.

In the testis, c-*kit* and *prm1* mRNAs are expressed in differentiating spermatogonia and round spermatids, respectively. Therefore, c-*kit* and *prm1* mRNAs were examined to determine the relationship between the survival of donor cells and donor-cell-derived progeny. In addition, at 2–3 weeks post-transplantation, a marked cell-specific increase in apoptosis was observed in meiotic spermatocytes (Fig. 4*C*). Apoptotic cells were not detected in control mice testes at 4 weeks following placebo injection (Fig 4*B*). Our data suggest that, for unknown reasons, most porcine donor cells, with a few exceptions, do not differentiate into post-meiotic spermatids, indicating that mouse Sertoli cells do not produce factors that fully support porcine spermatogenesis and spermiogenesis.

Previous studies demonstrated that donor spermatogonial stem-cell engraftment is enhanced when endogenous germ cells are absent because of genetic mutation or removal by ablative strategies. In addition, GnRH administration following germ-cell transplantation into busulfan-pretreated recipients leads to more effective colonization of donor-derived cells by suppressing endogenous spermatogenic events (Wistuba *et al.*, 2002). The best mouse-recipient model identified to date is the dominant *white spotting* (*W*) homozygous mutant male, which is congenitally infertile and lacks endogenous germ cells because of a mutation in the c-*kit receptor tyrosine kinase* (Yoshinaga *et al.*, 1991). Although GnRH administration to busulfan-pretreated mutant mice could serve as an efficient method for the colonizing of donor spermatogonial stem cells, commercially available *W* and *jsd* animals (Jackson Laboratory, Bar Harbor, ME, USA) are expensive, and complex breeding strategies are required to generate homozygous mutants (Nagano *et al.*, 2002). Furthermore, germ-cell-deficient mutants of other species are not available to serve as transplantation recipients. Therefore, we tested ICR male mice as recipients. Although they do not fully support spermatogenesis of xenografted porcine spermatogonia in their testes, they may provide a valuable experimental model for developing technologies that can be applied and evaluated in other species.

Although some treatments for specific cancers are more likely to result in subsequent infertility, it is very difficult to predict which children will be affected in later life (Choi *et al.*, 2004). Therefore, xenogenic spermatogonial transplantation may be an important method for protecting or restoring fertility at early stages. Although the effectiveness of interspecies spermatogonial transplantation is currently unclear, in the future, xenotransplantation of spermatogonia in clinical settings might be a genuine therapeutic possibility for childhood cancer survivors or endangered animal species at risk for infertility.

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