

Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts

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Retrieval, extracorporal storage and autotransplantation of testicular tissue could become an important strategy for preserving male gonadal function. The present study used syngeneic and immunodeficient *nude* mice as hosts, and immature and adult mice, neonatal and adult photoregressed Djungarian hamsters and neonatal marmosets to identify the potential of testicular tissue grafting to maintain the morphological and functional integrity of the testis. Testicular tissue was grafted s.c. either as fresh tissue or after cryopreservation into adult, orchidectomized hosts. The mice that received rodent testis tissue were autopsied 50 days later, and blood samples were collected. Sixty-five per cent of mouse isografts contained morphologically normal testicular tissue and seminiferous tubules with some degree of spermatogenic recovery. Mature spermatozoa were recovered after enzymatic disaggrega-

tion. Although the recovery of spermatogenesis was limited in adult mouse and hamster tissue, complete spermatogenesis was observed in grafts from immature rodents. Testicular tissue from neonatal marmosets developed up to the stage of spermatocytes at day 135 after xenografting. Androgen concentrations were comparable in intact control mice and in mice receiving fresh mouse and hamster grafts, slightly lower in mice receiving cryopreserved grafts and adult photoregressed hamster tissue, and low in castrated control mice and in mice receiving marmoset tissue. These results show that isografts and xenografts of immature and adult testicular tissue become functionally active as a s.c. graft in the mouse and that this approach might be useful in combination with cryopreservation as a tool for storage and activation of the male germ line and androgen replacement therapy in patients.

Introduction

The proportion of young adults that survive childhood cancer is approaching 1 in 250. High-dose chemotherapy and bone marrow transplantation have revolutionized the long-term remission or cure of aggressive tumours in young patients, but these treatments often cause permanent sterility as a result of the loss of spermatogonial stem cells (Apperley and Reddy, 1995; Naysmith *et al.*, 1998). Androgen deficiency due to the damage of Leydig cells is less frequently a problem. Although semen cryopreservation is practised routinely for adult males, there are no options at present for conserving fertility in prepubertal boys who receive sterilizing cancer therapy (Grundy *et al.*, 2001). However, several breakthroughs in the cryostorage of tissues as well as the transplantation of germ cells and reproductive organs have opened new pathways for preservation of fertility (Gosden and Nagano, 2002). Exciting new methods have been developed which could be used to restore fertility by the retrieval of gonadal tissue

before gonadotoxic therapy and retransplantation of germ-line stem cells or grafting tissue containing germ cells after patients have been cured of a malignant disease that does not involve the gonad. In females, some of the new techniques, such as the cryopreservation and grafting of ovarian tissue (Oktay *et al.*, 1998; Picton *et al.*, 2000; Wang *et al.*, 2002) or the culture and maturation of follicles *in vitro* (Murray and Spears, 2000), are already under investigation in clinical research trials (Radford *et al.*, 2001) and are being tested for their safety in clinical use (Kim *et al.*, 2001). In males, the advent of germ cell transplantation in mice (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994) and primates (Schlatt *et al.*, 1999, 2002), and culture of male germ cells *in vitro* (Tesarik *et al.*, 2000) present promising and innovative areas for scientific progress, but are still at an experimental stage (Schlatt *et al.*, 2000; Meachem *et al.*, 2001).

The present study describes the cryopreservation and subsequent grafting of mouse testicular tissue as an option for preserving the male germ line and generating mature spermatozoa. Furthermore, the ability of testicular tissue from newborn hamster and marmoset monkey tissue to differentiate after subdermal grafting into immunodeficient *nude* mice was analysed. In addition, the potential of

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grafting as a hormone replacement therapy after castration of the recipient was determined; this treatment might benefit a subset of patients suffering from androgen insufficiency after Leydig cell depletion.

Materials and Methods

Experimental surgery: murine isografts

The animal research protocol was licensed by the Home Office of the UK. First generation hybrid male mice were obtained by mating C57BL/6 × CBA/Ca in the animal colony at the University of Leeds. Testicular tissue was obtained from donor animals that were either 10 or 80 days old; recipients of isografts were 7 weeks old at the time of surgery.

Donor animals were killed by cervical dislocation and graft tissue was prepared by making an incision in the testicular capsules to expose the tubules. The size of the grafts was about 0.5–1.0 mm³, representing either a whole to a half of an immature testis or an equal size fragment of an adult testis. The tissues were maintained in sterile Leibovitz-L15 medium (Gibco, Paisley) at room temperature for up to 30 min, until the host was surgically prepared for grafting. Some grafts were selected at random for cryopreservation for transplantation up to 1 week later. The grafts were equilibrated for 20 min at 0°C in 1.5 mol dimethylsulphoxide (DMSO) l⁻¹, 0.1 mol sucrose l⁻¹ and 1% human serum albumin in medium and transferred to 1.5 ml cryovials (Nunc, Wiesbaden). The vials were loaded into a programmable freezer (Kryo 10; Planer Products, Sunbury) at 0°C and cooled at 2°C min⁻¹ to 7°C and then nucleated with cold tongs. Cooling proceeded at 0.3°C min⁻¹ to -40°C and then at 10°C min⁻¹ to -140°C. The vials were plunged into liquid nitrogen and transferred to a storage dewar. For thawing, the tubes were kept at room temperature for 1 min to evaporate any remaining liquid nitrogen in the tube and then swirled in a bath of water at 37°C for approximately 1 min. The contents were emptied immediately into a succession of Petri dishes that contained a descending gradient of DMSO (1.0, 0.5 and 0.0 mol l⁻¹, the first two steps containing albumin and sucrose as before) in Leibovitz medium for 3 min at each step to wash out the cryoprotectant.

Anaesthesia was induced in host animals with a halothane–oxygen mixture. A ventral mid-line incision was made in the skin and the body wall. The vasa deferentia were identified and the testes were exteriorized. The spermatic cord was ligatured and divided, and both testes were detached from adhering tissue and from the vasa efferentia. The body wall was closed. Two grafts were attached 2–3 mm either side of the mid-line using non-absorbable 6/0 prolene suture (Ethicon, Norderstedt) to secure their fibrous capsules. Finally, the skin wound was closed with Michel clips. A subset of animals was injected i.p. with a 2% (w/v) solution of bromodeoxyuridine (BrdU) at 100 mg kg⁻¹ at 2 h before autopsy.

The animals were killed by inhalation of CO₂ 50 days after the operation. A blood sample was collected termi-

nally via cardiac puncture and serum was prepared and stored at -20°C for subsequent assay of testosterone. An incision was made in the ventral skin to identify the grafts which were dissected for fixation. Fresh grafts from five adult animals were disaggregated to isolate live spermatozoa. The grafts were dissected and incubated for 30 min at 37°C in a rotating tube of 5 ml collagenase (Type I, 1 mg ml⁻¹; Sigma Chemicals, Deisenhofen) plus DNase (8 U ml⁻¹), and tissue remnants were pipetted through a fine tip to complete the disaggregation. The cell suspension was examined under phase-contrast microscopy.

Experimental surgery: xenografts

The animal research protocol was in accordance with the German Law on the Care and Use of Laboratory Animals and was licensed by the Regierungspräsident Münster. *Nude* male mice (HsdCpb:NMRI-nu; *n* = 31), 5–6 weeks of age, were purchased from Harlan-Winkelmann (Borchen). Testicular tissue was obtained from 4–8-day-old Djungarian hamsters (*Phodopus sungorus*, *n* = 12) or adult hamsters (*n* = 3) that were exposed to inhibitory photoperiods (8 h light:16 h dark) for 10 weeks to induce maximal regression of the testes (Schlatt *et al.*, 1995). Testis tissue was also retrieved from two newborn marmoset monkeys (*Callithrix jacchus*). Hamsters and monkeys were derived from institutional colonies of the Institute of Reproductive Medicine, University of Münster.

Adult hamsters were killed by CO₂ inhalation. The testes were decapsulated and dissected. Immature donors were killed by decapitation. The testes were dissected and cut into halves or quarters (hamster) or into 8–9 fragments (marmoset monkey). Fragments of tissue (about 0.5–1.0 mm³) were dissected and maintained for up to 30 min in ice-cold sterile Dulbecco's modified Eagle's medium (Gibco).

Anaesthesia was induced in *nude* mice using Avertin (0.63 g kg⁻¹). First, the animals were castrated through two scrotal incisions. The spermatic cord was ligatured and the testes were removed. The scrotal skin was closed using Michel clips. Four incisions of 4–5 mm either side were made along two lines 3–4 mm of the mid-line. Eight grafts were placed into each host and secured using non-absorbable 6/0 prolene suture (Ethicon) to the muscle layer of the skin on the dorsal surface either side of the mid-line. The wounds were closed with Michel clips. Nine mice were castrated and sham-operated, and another ten mice were left intact and sham-operated to serve as a control group.

Of the 12 *nude* mice that received xenografts, three mice died as a result of infections that occurred during housing in the animal house between day 30 and day 40 after grafting. Four mice with newborn hamster grafts, two mice with grafts from adult photoregressed tissue and three mice with grafts from neonatal marmosets survived until they were killed and were in good condition at the end of the experiment. The mice carrying hamster grafts were killed by CO₂ inhalation 50 days after the operation. The first mouse

to receive monkey testicular tissue was killed after 100 days and the remaining two mice were killed 135 days after the grafting procedure. The last two mice received injections of gonadotrophins (Pergonal; Serono Pharma, Unterschleissheim three injections of 1.5 iu per week) for the last 35 days. The body weight and the mass of the seminal vesicles were recorded. An incision was made in the ventral skin to identify the size and number of grafts that were dissected for fixation and histological analysis.

Microscopy

Tissue was fixed in Bouin's fluid, embedded in paraffin wax and then cut into serial sections of 5 μm in thickness. Sections were stained with haematoxylin and eosin. Specimens from BrdU-treated animals were stained immunohistochemically. In brief, paraffin wax was removed from the sections and they were rehydrated. After rinsing with tap and distilled water, sections were hydrolysed using 1 mol HCl l^{-1} at 70°C for 8 min in a temperature-controlled microwave oven. After washing in running tap water, the sections were incubated for 15 min at room temperature in 0.1% (w/v) trypsin in Tris-buffered saline (10 mmol TBS l^{-1} , 150 mmol NaCl l^{-1} , pH 7.6). Non-specific staining was blocked for 20 min immediately before incubation with the monoclonal mouse anti-BrdU IgG (DAKO M0744, diluted 1:30 in TBS + 0.1% BSA, 60 min or overnight) by using 5% normal goat serum. After three washes in TBS, the second goat anti-mouse IgG linked to colloidal gold was incubated for 60 min. After several washes in TBS and distilled water, silver enhancement was performed until a dark brownish precipitate became visible by microscopic observation. The reaction was stopped by rinsing in distilled water. Slides were then counterstained with haematoxylin, dehydrated and mounted.

The slides were coded and the degree of spermatogenesis was assessed blindly using a random systematic approach. Two sections of each graft were chosen for analysis. All seminiferous tubules that were observed in the samples were scored. The seminiferous tubules were sorted into one of the following categories: (i) complete tubular atrophy and Sertoli cell only tubules; (ii) spermatogonia but no other germ cells present; (iii) spermatocytes as the most advanced germ cells; (iv) round spermatids as the most advanced germ cells; (v) elongated spermatids as the most advanced germ cells; and (vi) mature spermatozoa present in the lumen of the tubule.

Small pieces of tissue (< 1 mm^3) were fixed overnight in 2.5% (w/v) glutaraldehyde in cacodylate buffer for 3 h. After several washes in cacodylate buffer, the tissues were post-fixed in 1% (w/v) osmium tetroxide for 2 h and subsequently dehydrated in a gradient of ethanol. Tissues were infiltrated with LR White acrylic resin (London Resin Co., London) and polymerized at 60°C. Ultrathin sections (50–60 nm in thickness) were cut and mounted on nickel grids, and stained with 4% (w/v) aqueous uranyl acetate and Reynold's lead citrate. The grids were examined under a

transmission electron microscope (JEOL 100S; JEOL, Tokyo) at magnifications ranging from $\times 30\,000$ to $\times 100\,000$.

Radioimmunoassay for testosterone

Each serum sample was measured in duplicate and extracted with diethyl ether. The assay was carried out using an antiserum raised against testosterone-3-BSA conjugate with tritiated testosterone as the label. Unbound testosterone was separated by adsorption on to dextran-coated charcoal. Crossreactivity with dihydrotestosterone was 53%, but < 5% with other androgens. Interassay variation was 7–10% over the range of 1–25 nmol l^{-1} . The data were compared statistically using ANOVA and the Student's *t* test.

Results

Histological evaluation of spermatogenic recovery

Grafted testicular tissue was recovered s.c. from all host mice. Recovery was facilitated by identification of the grafting sites through non-absorbable sutures even where there was partial or complete overgrowth of the graft by connective tissues. In mouse to mouse grafts, photoregressed hamster and monkey grafts, the recovered tissue specimens were too small to be weighed accurately, although they appeared slightly smaller than when they were fresh. Grafts from neonatal hamster tissue grew much larger (> 10-fold) compared with the size of testicular fragments used for grafting.

Evaluation of spermatogenesis in mouse isografts

Typical examples of the histology observed after isografting are shown (Fig. 1a–e). The testicular tissue was always partially, and often completely, embedded in the skin tissue (Fig. 1a). The interstitium contained morphologically normal blood vessels and Leydig cells (Fig. 1d). The seminiferous tubules showed variable degrees of spermatogenic activity ranging from complete atrophy of the tubules to full spermatogenesis (Fig. 1b–e). An analysis of the most advanced type of germ cell observed in all the implanted and recovered grafts is shown (Table 1). Two-thirds of the grafts, whether from immature or mature donors, showed some degree of spermatogenic recovery, as indicated by at least one seminiferous tubule with developing germ cells. The morphological appearance of Sertoli, Leydig and germ cells from all stages was normal. All treatment groups were similar in their potential to recover spermatogenesis up to the stage of spermatids. There was no obvious adverse effect after cryopreservation of the tissue. In animals injected with bromodeoxyuridine, immunohistochemical labelling revealed a large number of labelled spermatogonia and preleptotene spermatocytes, indicating intense proliferative activity of early germ cells (Fig. 1f).

Ultrastructural analysis of the germ cells confirmed the normal morphological appearance of the germ cells in the grafts (Fig. 1g). Morphologically normal spermatozoa were retrieved from the grafts, indicating the opportunity to perform assisted fertilization using these gametes (Fig. 1h).

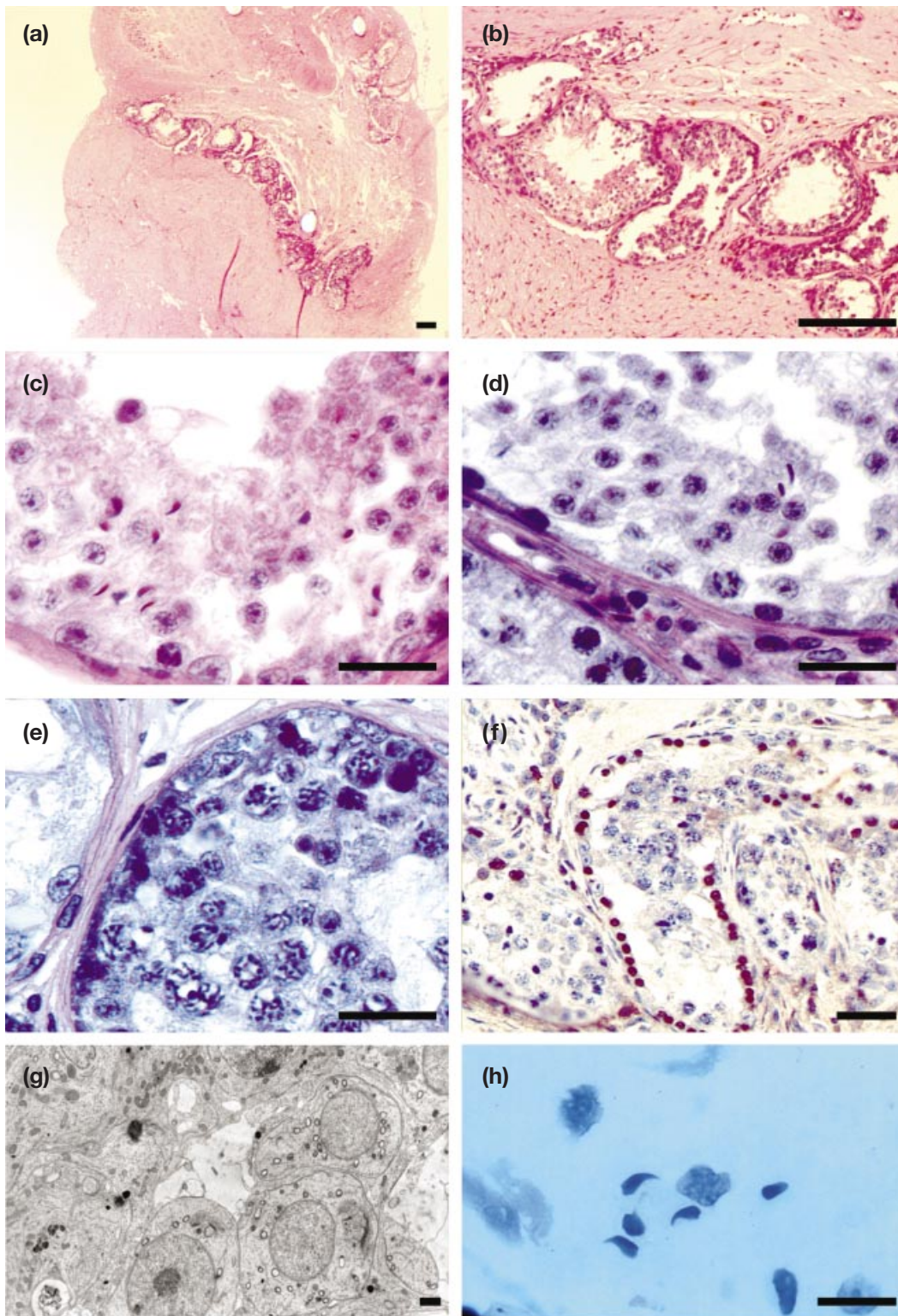


Fig. 1. Representative histological micrographs illustrating the growth and recovery of mouse testicular tissue as a s.c. isograft. (a) Low power view showing the location of the graft in the skin. The graft was derived from a 10-day-old mouse without cryopreservation and has been completely overgrown by the connective tissue of the skin. (b) Higher magnification of (a). The seminiferous tubules show different degrees of spermatogenic activity. (a,b) Haematoxylin–eosin stained. (c) Higher magnification of (a) and (b). A seminiferous tubule with qualitatively normal spermatogenesis up to the stage of elongated spermatids. (d) A seminiferous tubule from

Table 1. Most advanced stage of germ cell development in testicular isografts: effect of donor age and cryopreservation on spermatogenesis in a total of 31 grafts recovered from murine hosts

Donor age	Cryopreservation	Number of grafts	Tubule atrophy	Spermatocytes	Spermatids	Spermatozoa
Immature	No	4	0	2	2	0
Immature	Yes	12	2	6	4	0
Mature	No	4	3	1	0	0
Mature	Yes	11	2	3	3	3

In the grafts from immature testis tissue, all stages of germ cell maturation were observed in a given seminiferous tubule up to the stage of the most advanced germ cell. However, in the grafts recovered from adult tissue, there were often only germ cells of the most advanced germ cell stage with no or few immature stages.

Evaluation of spermatogenesis in xenografts

Grafted tissue from hamster and marmoset monkey tissue survived as a xenograft (Fig. 2). Of the 32 grafts from newborn hamster tissue, 30 grafts were recovered. All grafts had grown from half the size of a newborn hamster testis to about half the size of an adult hamster testis. The testicular structures were clearly visible in the back of the hosts before dissection. The seminiferous tubules in newborn hamster grafts showed various degrees of spermatogenesis (Table 2). Frequently, the testicular tissue had fully differentiated and completed spermatogenesis up to the stage of mature spermatozoa, which were observed in the seminiferous tubules of > 25% of the grafts (Fig. 2b; Table 2). In contrast, only half of the adult photoregressed hamster tissue grafts survived and only two of the eight recovered grafts showed some degree of spermatogenic recovery (Fig. 2c; Table 2). When neonatal testicular tissue from marmoset monkeys was used for grafting, seven of 18 testicular grafts were recovered. In the two grafts recovered 100 days after grafting, the testicular tissue showed some degree of differentiation as the Sertoli cell nuclei showed a regular orientation along the basement membrane of the cords, gonocytes had disappeared and spermatogonia were the most advanced types of germ cell and in mitosis. Although no major histological changes were observed after gonadotrophin stimulation at 135 days after grafting, primary spermatocytes were observed as the most advanced types of germ cell in all five grafts recovered in this group.

Evaluation of androgen production in grafted animals

In all orchidectomized hosts from which mouse to mouse grafts were recovered, serum androgen concentrations indicated that hormone secretion had occurred. The group of mice that received immature grafts showed testosterone concentrations similar to the group of intact control mice. Slightly lower concentrations of testosterone were observed in animals that received grafts from fully developed testes (Table 3). However, all groups that received mouse grafts showed significantly higher concentrations of androgens in comparison with the castrated control animals.

As serum testosterone concentrations are quite variable in mice, androgen production in xenografts was analysed by determination of seminal vesicle mass which presents a more integrated indicator of androgen action. In control groups of *nude* mice, the average mass of the seminal vesicles was 233.2 ± 40.5 mg ($n = 10$) in intact and 18.5 ± 2.6 mg ($n = 9$) in castrated animals. Mice grafted with neonatal hamster testes showed seminal vesicle masses in the normal range (200 ± 30.7 mg, $n = 4$). The two mice that received grafts from photoregressed hamster testis tissue had seminal vesicle masses of 28.7 and 100 mg, indicating a low production of androgens from the grafts. None of the three mice that received marmoset monkey tissue as grafts showed seminal vesicle masses above that of the castrated range (4–18 mg).

Discussion

The present study revealed that grafting of immature mouse and hamster and adult mouse testicular tissue into immunodeficient hosts leads to the initiation and restoration of spermatogenesis and steroidogenesis. It was also demonstrated that cryopreservation of mouse tissue before grafting is

a cryopreserved immature graft containing mostly mature types of round and elongating spermatids with only a few spermatocytes. The peritubular cells and the interstitium, including Leydig cells and blood vessels, appear to be morphologically normal. (e) A testicular area containing seminiferous tubules with no germ cells present next to a tubule with spermatogenesis up to the stage of pachytene spermatocytes. The tissue was derived from an adult cryopreserved graft. (c–e) Periodic acid–Schiff staining. (f) Same graft as shown in (e). Localization of bromodeoxyuridine in many early germ cells indicates a high level of germ cell proliferation in seminiferous tubules arrested at the stage of spermatocytes. (f) Immuno-gold–silver staining of bromodeoxyuridine, counterstain haematoxylin. (g) Electron microscopic observation of germ cells in adult, cryopreserved grafts revealed normal ultrastructural appearance of mature germ cells. Ultrathin section contrasted by lead citrate. (h) Cytospin preparation of the grafted and enzymatically dispersed tissue from grafting a fresh adult organ reveals the presence of mature spermatozoa in the samples. Scale bars represent (a,b) 100 μ m, (c–e) 20 μ m, (f) 50 μ m, (g) 1 μ m and (h) 10 μ m.

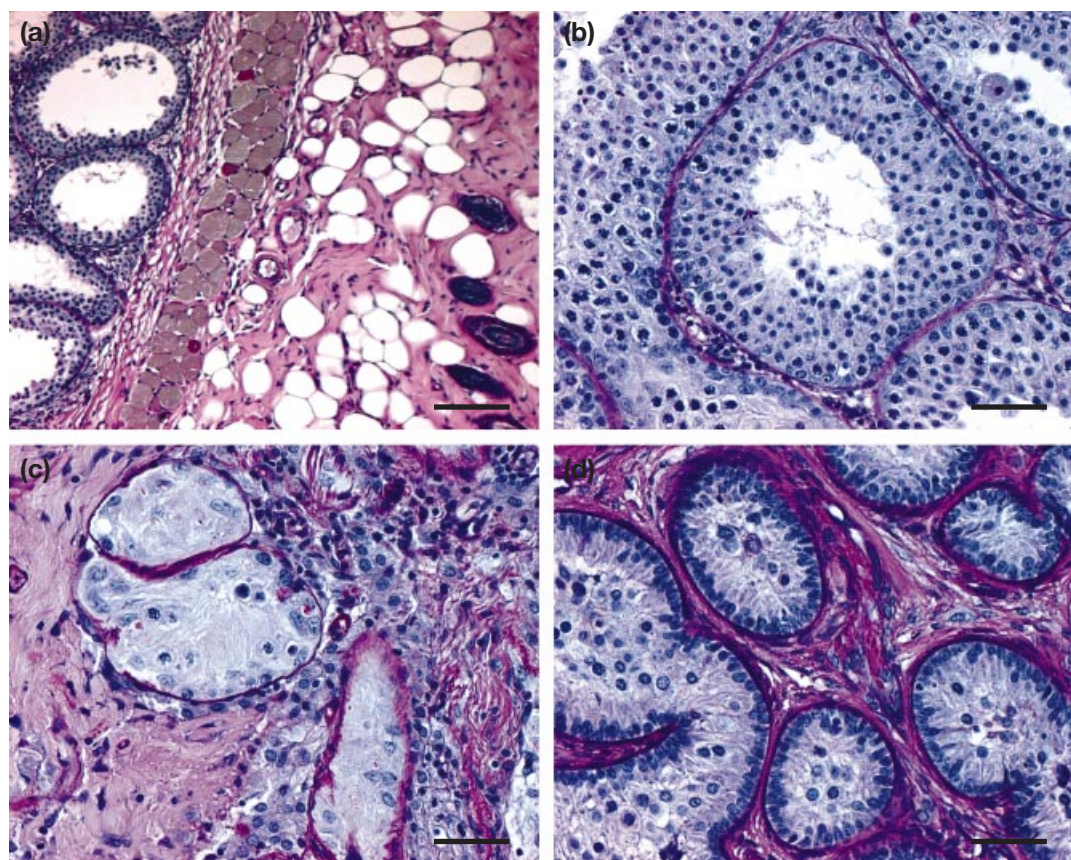


Fig. 2. Representative histological micrographs showing the development of xenografts from (a,b) newborn and (c) photoregressed Djungarian hamsters and (d) newborn marmoset monkey in the back skin of immunodeficient *nude* mice. (a) Low power view showing the mouse skin tissue with hair follicles, fat and muscle layers. Fully differentiated testicular tissue is observed attached directly to the muscle layer. (b) A high power view of the same graft as shown in (a). In some tubules, quantitatively normal spermatogenesis is observed with all stages of germ cells present. (c) Grafted tissue from photoregressed hamster tissue showed much less capacity. The most advanced progression is to spermatocytes. Many tubules degenerated and infiltration of immune cells is regularly observed. (d) Marmoset monkey tissue differentiated and spermatogenesis was initiated to early spermatocytes. Scale bars represent (a) 125 μm and (b–d) 50 μm .

possible, which allows the maintenance and storage of testicular tissue for indefinite periods. The collection of mature male gametes several weeks after grafting opens another option for germline storage that could be useful in experimental biology, applied biology and medicine. For instance, it provides a new strategy for fertility protection in cancer patients as well as storage of testicular tissue for generating mature gametes in valuable livestock or endangered species (Schlatt *et al.*, 2000).

The cytology showed that all aspects of rodent spermatogenesis could be initiated after grafting when immature testis tissue was used. The tubules showed mixed degrees of spermatogenic recovery up to the status of qualitatively normal spermatogenesis with all stages of germ cells. This finding indicates that spermatogenesis has been initiated from stem cells and that in grafts from immature donors, spermatogenesis appears to occur according to the kinetics of the donor.

Adult mouse and adult photoregressed hamster testis

tissue were partially able to recover function after grafting, but usually showed signs of severe atrophy. There was no obvious explanation for this difference. However, mature spermatozoa could be retrieved from mouse tissues, demonstrating that it is possible to recover gametes after cryopreservation and grafting. It is interesting to note that the re-initiation of spermatogenesis in adult tissue was not comparable to the outcome after grafting of immature tissue. Although some seminiferous tubules in mouse grafts contained spermatozoa, it seemed as if these spermatozoa derive from germ cells which completed the differentiation process after grafting of the tissue. Only very few stem cells were able to restart spermatogenesis after grafting of adult mouse tissue. Similarly, adult photoregressed hamster tissue did not recover well and most of the tissue degenerated. This finding indicates that the ability to obtain spermatogenic recovery after grafting is much more promising using immature testicular tissue. As spermatogenesis in the hamster testis was fully regressed, the reason for the difference

Table 2. Most advanced stage of germ cell development in a total number of 45 testicular xenografts recovered from murine hosts

Donor	Reproductive status	Number of grafts	Tubule atrophy	Spermatocytes	Spermatids	Spermatozoa
Hamster	Neonatal	30	3	9	10	8
Hamster	Regressed	8	6	2	0	0
Marmoset ^a	Neonatal	5	0	5	0	0

^aThese results represent the two *nude* mice analysed at day 135 after grafting and at day 35 of gonadotrophin treatment. Two more grafts were recovered from one mouse 100 days after grafting, showing that actively dividing spermatogonia were the most advanced germ cells.

Table 3. Testicular tissue transplantation: effects of age of donor and cryopreservation on serum testosterone concentrations in murine hosts

Experimental group	Serum testosterone (nmol l ⁻¹)
Intact controls	6.5 ± 2.1
Castrated controls	0.3 ± 0.3*
Fresh immature grafts	7.1 ± 1.4
Cryopreserved immature grafts	5.1 ± 1.5
Fresh mature grafts	2.7 ± 0.7
Cryopreserved mature grafts	3.6 ± 0.6

Values are mean ± SEM.

*Significantly lower than all other groups ($P < 0.001$) ($n = 5-10$ per group).

between immature and adult tissue cannot be exclusively related to the status of spermatogenesis and the activity of the testicular tissue at the time of grafting, but is most likely due to differences in the ability of the immature tissue to survive periods of ischaemia or the possibility that immature tissue is more effective for angiogenesis in the host.

Although the seminiferous tubules in grafts from monkey tissue showed some differentiation, the most advanced stage was meiotic germ cells. As the pattern of differentiation was quite similar among all five grafts, it appears that the period of 135 days after grafting was not sufficient to induce more differentiated germ cells. Whether the appearance of spermatocytes between day 100 and day 135 was dependent on the stimulation with gonadotrophins or reflects a hormone-independent further development during the additional 1 month cannot be answered from the present study. The initiation of full spermatogenesis in grafts from immature donors renders it highly useful for the generation of male gametes in patients or animals in which both testes have to be removed or testicular function is completely abolished by medical intervention or disease. Furthermore, grafting of testis tissue from different species permits the design of studies to determine the effects of hormones and other stimuli on testicular differentiation.

DMSO was used as the cryoprotectant, although glycerol and ethylene glycol are similarly effective for the preservation of testicular cells (Brook *et al.*, 2001). Furthermore, testicular stem cells have been cryopreserved in glycerol-containing medium using a rapid freezing approach before retransplantation into a mouse testis for recolonizing the host seminiferous tubules and re-initiating spermatogenesis

(Avarbock *et al.*, 1996). These findings indicate that testicular tissue and male germ line stem cells are quite tolerant of cryopreservation, and this step in the procedure may be less critical than the preparation of ovarian tissue for grafting (Oktay *et al.*, 1998; Picton *et al.*, 2000).

The results from the present study confirm the observations made by Deansley (1954). Using the crude cryotechnology available at the time (cooling in glycerol to -79°C), Deansley was able to demonstrate initiation of spermatogenesis in rat testicular allografts. As in the present study, vascular anastomosis could not be used. However, leucocytic infiltration occurred and this interfered with the restoration of graft activity as inbred strains for isogenic combinations of donor and host were not available at the time.

Leydig cells appeared morphologically and functionally normal. Neither hypotrophy nor hypertrophy of Leydig cells was observed in the grafts. Immature donor tissue was used and testosterone concentrations estimated from serum or indirectly by the mass of the seminal vesicles were at physiological values 50 days after grafting. Testosterone concentrations were restored in grafted animals using rodent tissue, indicating that the procedure could serve as an androgen replacement therapy. In this respect, it is interesting to note that the androgen contents were normal in the castrated mice that received grafts from immature testicular tissue.

The decrease of steroid production during ageing of Leydig cells is a controversial topic and reasons for ageing of the steroid-producing tissue have been under intense investigation (Syntin *et al.*, 2001; Chen *et al.*, 2002; Culty *et al.*, 2002). Chen *et al.* (1996) showed that androgen

deficiency of aged brown Norway rats is restored after depletion and restoration of Leydig cells. The results of the present study might be of relevance to this issue as grafting might present an elegant tool to address some of the open questions. Steroidogenesis was fully restored in immature hamster and mouse testes, but only partially restored in adult testes from intact mice and photoregressed hamsters. In conclusion, Leydig cells from young testes have a higher capacity than adult Leydig cells to repopulate the interstitium and to differentiate from precursors. The age-dependent ability to restore Leydig cells might be the reason for the age difference in the capacity to (re)initiate spermatogenesis after grafting. A poor recovery of Leydig cells may hinder a full recovery of spermatogenesis. As androgen replacement therapy in the hypogonadal ageing male receives increasing attention (Basaria and Dobs, 2001; Vermeulen, 2001), grafting of testicular tissue might become an interesting experimental tool for the study of ageing Leydig cell function and for the development of new therapeutic strategies. Grafting of testis tissue of different ages in combination with hormone treatment might become an elegant strategy to explore the potential of Leydig cells to recover and regain normal function. Surprisingly, the grafts from immature monkey tissue did not produce any androgens independent of the stimulation with human gonadotrophins. The Leydig cells in the graft did not appear to be responsive to gonadotrophins from mice or humans, indicating that the period of 100–135 days after grafting was not sufficient to induce maturation of the interstitial tissue.

In conclusion, the present study demonstrates the use of testicular tissue cryopreservation and grafting as a tool for preserving the male germ line, generating mature male gametes from immature and mature gonads, and replacing testosterone in castrated recipients. Thus, this strategy can serve to restore the dual functions of the organ – spermatogenesis and hormone production.

The authors thank S. Matthews for assistance with surgery and the animal care staff in the Department of Biology, University of Leeds. The technical assistance of I. Upmann and R. Sandhowe-Klawerkamp is gratefully acknowledged. This work was supported by a joint grant to R. Gosden and S. Schlatt from the German Academic Exchange Service (DAAD) and the British Council.

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Received 13 March 2002.

First decision 22 May 2002.

Revised manuscript received 17 June 2002.

Accepted 18 June 2002.