Complete Spermatogenesis in Orthotopic But Not in Ectopic Transplants of Autologously Grafted Marmoset Testicular Tissue

C. Marc Luetjens, Jan-Bernd Stukenborg, Eberhard Nieschlag, Manuela Simoni, and Joachim Wistuba

Institute of Reproductive Medicine of the University, 48149 Münster, Germany

Testicular grafting has the potential to become a method to preserve fertility in prepubertal boys undergoing cancer treatment. The possibility of successful germ cell maturation after autologous grafting should be proven preclinically in a nonhuman primate model. Therefore, in two experiments, we analyzed the potential of autologous testicular grafting in the marmoset model. A first experiment in immature and adult semi-castrated monkeys addressed the question of whether full spermatogenesis in an ectopic graft could be achieved under a relatively normal endocrine milieu and whether the donor's age is of influence. A second experiment in castrated immature animals examined whether the transplantation site [ectopic (back skin) or orthotopic (scrotum)] influences spermatogenic progress and whether cryopreserved tissue can be successfully transplanted. Grafts were analyzed by histology, immunohistochemistry, and morphometry. Bioactive chorionic gonadotropin and serum testosterone were measured. In the adults, ectopic grafts degenerated, whereas in the immature animals, grafts survived at the spermatogonial level. In the castrates, none of the cryopreserved grafts survived, ectopic grafts were meiotically arrested, but orthotopic transplants completed spermatogenesis. Androgen and bioactive chorionic gonadotropin levels were not decisive for graft development. When ectopic and orthotopic transplantation sites were compared, the scrotum has a substantially lower temperature. Thus, the higher temperature at the ectopic transplantation site may contribute to spermatogenic arrest. Autologous grafting of nonhuman primate testicular tissues can result in complete spermatogenesis. Our findings indicate that transplantation site and developmental age of the tissue play a role more important than the endocrine milieu. (Endocrinology 149: 1736–1747, 2008)
maintain high local testosterone concentrations (see below) (2, 4, 6, 7). In a previous experiment, we showed that testicular tissue transplanted heterologously degenerates completely, whereas testis grafts transplanted autologously under the back skin of castrated immature animals survived for a long period and matured up to the meiotic stage before spermatogenesis was arrested (10).

The present study was aimed to understand and overcome meiotic arrest in autologously grafted material in this non-human primate model. Therefore, we conducted two experiments. The first experiment was performed in adult and immature hemi-castrated monkeys and addressed the question of whether spermatogenesis in an ectopic graft could be achieved in the presence of a relatively normal male hormonal milieu and whether transplantation success is influenced by the donors’ age. The second experiment performed in fully castrated immature animals examined whether the site of transplantation influences the outcome of spermatogenesis and whether cryopreserved testis material obtained before puberty can be successfully transplanted.

Materials and Methods

Experimental design

In total, the experimental setting comprised four treatment groups with five marmosets each (Callithrix jacchus; n = 20). In the first experiment, one group of hemi-castrated adult animals (aged 21 months) was compared with a group of hemi-castrated immature animals (aged 4 wk). In the second experiment, two groups of immature animals were analyzed (Fig. 1).

Hemi-castrated animals, experiment 1: endocrine milieu and age

In group 1, immature animals were hemi-castrated, and the testicular tissue was divided into fragments that were either ectopically grafted under the back skin (four transplants per animal) or fixed as pregraft control tissue. The contralateral testes remained to ensure normal hormonal milieu and whether transplantation success is influenced by the donors’ age. The second experiment performed in fully castrated immature animals examined whether the site of transplantation influences the outcome of spermatogenesis and whether cryopreserved testis tissue obtained before puberty can be successfully transplanted.

Fully castrated animals, experiment 2: transplantation site and cryopreservation

In group 3, immature animals were castrated, and fragments of testicular tissue were grafted either ectopically under the back skin (four transplants per animal) or orthotopically into the emptied scrotum (four transplants per animal). Fragments of each testis were fixed as pregraft controls. Tissues were explanted 14 months after transplantation and evaluated as described. This approach aimed at identifying the influence of the transplantation site on the grafting success.

Animals, tissue collection, and transplantation procedure

Testes were dissected from immature (n = 15, age 1 month) and adult (n = 5, age 21 months) marmosets (C. jacchus) obtained from the institutional breeding facilities. The monkeys were anesthetized with Saffan during surgery (Provet AG, Lyssach, Switzerland; 0.1 ml/100 g body weight). Castration was performed through scrotal incisions, and subsequently, the scrotal skin was stitched after removal of the testis. Testicular fragments (size 0.5–1 mm3) were kept in ice-cold DMEM (Invitrogen GmbH, Karlsruhe, Germany) for up to 20 min until grafting (see Ref. 10). For ectopic grafting, skin incisions of 4–5 mm were made on either side of the dorsal midline, and four grafts per recipient were autologously placed underneath the shaved back skin. The scrotal grafts were placed in the opening formed during castration before the scrotum was closed. Incisions were sewed with a single suture. Cryopreserved testis tissue was grafted after storing and thawing by an identical procedure 4 months after the first surgery. Three of the grafts were selected at random for cryopreservation according to the protocol in Ref. 2 for transplantation up to 4 months later. The grafts were equilibrated for 1 h at 4°C in 1.5 M dimethylsulfoxide, 0.1 M sucrose, and 1% human serum albumin in medium and transferred to 0.25-mL straws (type CBS; Consarct GmbH, Schöllkrippen, Germany). The straws were loaded into a programmable freezer (Kryoautomat Consarct BV-65; Consarct) at 4°C and cooled at 2°C/min to −7°C and then nucleated with cold tongs. Cooling proceeded at −0.3°C/min to −40°C and then at 10°C/min to −140°C. The straws were plunged into liquid nitrogen and transferred to a storage Dewar flask. For thawing, the straws 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 dimethylsulfoxide (1.0, 0.5, and 0.0 M, each step containing 1% human albumin and 0.1 M sucrose) in DMEM for 3 min at each step to wash out remaining cryoprotectant.

Throughout the experimental period, animals were kept within their family groups, receiving normal diet and water ad libitum. They were regularly weighed, and blood samples (500 μL per sample) were drawn over the experimental period. For graft analysis, the animals were anesthetized with Saffan (0.2 ml/100 g body weight) and killed by exsanguination. Body weight was recorded, and blood was collected and stored at −20°C. The back skin was removed, and testicular grafts were dissected from the back skin and the scrotum. In groups 1 and 2, the remaining right testis and epididymis were removed. The experimental work was performed in accordance with the German Federal Law on the Care and Use of Laboratory Animals (license no. G32/2005).

Sample preparation for histological evaluation

Testicular and pregraft fragments were obtained for control purposes at castration, and the explanted grafts were individually fixed in Bouin’s solution for 4 h or snap frozen in liquid nitrogen and stored at −80°C. Tissues were routinely embedded in paraffin, cut into sections (5 μm), and stained with periodic acid Schiff, hematoxylin.

To assess spermatogenic progress and graft development, we used a previously established scheme (7, 10). In brief, tubular cross-sections lacking germ cells were determined as Sertoli-cell-only (SCO) tubules, and the most advanced germ cell type in each graft was used to describe spermatogenic progress. All seminiferous tubules of a cross-section were analyzed morphometrically in the most central cross-section of each graft and in material from 4-wk-old testes as well as from the contralateral testis of the hemi-castrated animals of groups 1 and 2. Pieces of the control tissue were taken from the central portion of the testes. The size of the fragment and the number of tubular cross-sections examined was similar to those of the transplants recovered from the experimental animals. The size of the seminiferous lumen and tubular diameter were analyzed with an Axioskop microscope (Zeiss, Oberkochen, Germany) and Axiovision 4.1 (Zeiss) software. Representative images were taken at magnifications of ×10, ×25, and ×40 (AxioCam, Zeiss).
Immunohistochemistry

Only primary antibodies validated for Callithrix tissues were used (10). BOULE (boule-like) protein in the sections was analyzed by a rabbit polyclonal antibody (diluted 1:300) (29, 30) and CDC25A by a rabbit polyclonal antibody (1:50, sc-97; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). All antibodies were applied for 60 min at room temperature in a blocking buffer. For all staining, Dako-LSAB 2 System (K0672; Dako Diagnostika, Glostrup, Denmark) was added for 30 min after washing, followed by an additional washing step and incubation with diaminobenzidine (DAB) (Dako Diagnostika) for 20 min. Antibodies were visualized by a secondary horseradish peroxidase-labeled mixed antiamouse and antirabbit IgG (1:100 dilution, K0672; Dako Diagnostika). DAB, used as a substrate, produced a dark brown signal. Briefly, the staining process was as follows. After washing, the slides were incubated in 3% (vol/vol) hydrogen peroxide to suppress endogenous peroxidase activity. After washing in Tris-buffered saline buffer (10 mM Tris and 150 mM NaCl, pH 7.6), nonspecific background was blocked by incubation in 5% (vol/vol) normal goat serum diluted in incubation buffer [0.1% (wt/vol) BSA in washing buffer]. The primary antibody was incubated in incubation buffer for more than 90 min at room temperature. After extensive washing, the secondary antibodies were added and incubated as a cocktail for more than 90 min at room temperature. DAB was finally added for 8–10 min followed by several washing steps. Control steps were performed by omitting the primary antibody on adjacent sections. In a final step, the slides were counterstained with hematoxylin for 10 sec and mounted under coverslips with Dako Faramount (Dako Diagnostika) before observation using a microscope (Axioskop, Zeiss) at different magnifications (objectives, ×10 and ×25).

Digital images of equal exposure were acquired with a CCD camera (Axiocam, Zeiss) controlled by image software (Axiovision, Zeiss).
Hormone measurements

Serum testosterone levels were measured using a previously published RIA (25). Each sample was processed in duplicate after extraction with diethyl ether. The intra- and interassay coefficients of variation were 10.3 and 11.5%, respectively. The detection limit of the assay was 0.68 nmol/liter.

The reproductive endocrinology of the neotropical primates differs from that of other primates owing to peculiarities in the function of the LH/chorionic gonadotropin (CG) system. The marmoset monkey does not express the β-subunit of LH but does express the β-subunit of CG. This is the only gonadotrophin with LH activity in this species (27, 31). Serum bioactive CG (BioCG) levels of the last three sampling dates were measured by an in vitro bioassay based on murine Leydig cells according to a previously established method for bioactive LH measurement (28, 32) and verified in several studies for male marmosets (10). The intra- and interassay coefficients of variation were 7.6 and 13.0%, respectively. The detection limit of the assay was 5.3 U/liter (WHO 78/549).

Thermography

Temperature measurements of the shaved back skin and the furless scrotum were performed on three different untreated animals. We used an infrared camera (Variotherm; Jenoptik AG, Jena, Germany) controlled by image software (Irbis; Jenoptik).

Statistical analysis

Data were analyzed by applying one-way ANOVA. Values of tubular and luminal diameter were compared by ANOVA (all pairwise, multiple comparison by Tukey test). Computations were performed using the statistical software package SIGMASTAT 2.03 (SPSS Inc., Chicago, IL). Values were considered significantly different if P < 0.05. Analysis of the testosterone and BioCG values were performed for testosterone by univariate variance analysis for repeated measurements over the entire experimental period and for BioCG over the last three blood sampling dates.

Results

Body weight gain over the study period observed in both experiments

Groups 1, 3, and 4: immature animals. The body weight of all animals increased over the study period (Table 1). At the age of 4 wk, the marmosets had an average weight of 67.3 ± 2.8 g. After surgery, they were housed within their families. Twelve weeks later, when the immature animals reached a sufficient developmental state (225.2 ± 8.0 g mean body weight), consecutive blood samples up to the end of the study period were drawn twice weekly. The average body weight at the end of the study period was 317.9 ± 13.9 g. The average weight gain in the 60 wk of the study did not vary significantly among the three animal groups (Table 1).

Group 2: adult animals. The monkeys had an average body weight of 408.4 ± 20.1 g at the time of surgery and were kept within their families after surgery for another 26 wk. No significant weight change (425.6 ± 14.0 g) was observed over the study period.

The significant weight difference among the animals of groups 1, 3, and 4 compared with group 2 at the end of the study is caused by the different age at the end of the study period (groups 1, 3, and 4 were about 65 wk; group 2 was about 90 wk).

Graft survival, spermatogenic progress, and endocrine state

In all 20 treated animals, the grafts and remaining testes and epididymides were explanted and weighed, and the number of surviving grafts was recorded. No significant weight differences among the analyzed grafts were found, nor were significant organ weight differences found between the groups. The grafts were analyzed for the stage of spermatogenic progress (Table 1). In none of the analyzed grafts were any signs of inflammation found, such as macrophages or other leukocytes.

Hemi-castrated animals, experiment 1: endocrine milieu and age

Group 1. In total, nine grafts of 20 transplanted fragments were recovered from four individuals (Table 1). In one mon-
key, no graft survived. In the grafts, spermatogonia were observed as the most advanced germ cell stage (28.7 ± 30.9% of the tubules; Table 2). In the pregraft control tissues, only gonocytes positioned in the tubular lumen and a few early spermatogonia at the basal membrane were observed. The mature contralateral control testis showed complete spermatogenesis up to the level of elongated spermatids in almost every tubular cross-section (Table 2). Thus, the grafted material differentiated only up to spermatogonial arrest. Occasionally, in the ectopic grafts, seminiferous tubules lacking all germ cells (SCO) were observed (53.4 ± 27.1% of tubules; Table 2). On average, serum testosterone levels had already risen over 20 nmol/liter during the sampling period but remained very variable until shortly before the end of the study (Fig. 2A).

Group 2. Seven ectopic grafts were explanted from four animals (Table 1). In one animal, none of the grafted fragments survived the study period. In general, the ectopic grafts explanted from the back skin sites contained fibrotic or SCO tubules that had lost all germ cells. In only one ectopic graft, a single tubule with some degenerating spermatocytes (0.4 ± 1.2% of the tubules of the section; Table 2) was found. All other tubules of this transplant exhibited only spermatogonia as the most advanced germ cell type or were SCO. As expected, the average testosterone levels remained normal over the entire sampling phase (Fig. 2A).

Serum testosterone values of group 2 were also significantly elevated (P < 0.001) compared with the postpubertal levels of the semi-castrated monkeys of group 1. Both groups had similar testosterone values only in the last six study weeks (Fig. 2A).

The different ages of the monkeys at the time point of transplantation resulted in different exaptation rates (in immature animals, 45% of the grafts were found at study end vs. 35% in the adults). Although in only 20% (one of five) of the adult animals did the transplanted material contain germ cells, in 60% of the juvenile animals, grafts with spermatogonia were found. The majority of the tissue grafted in the adult state degenerated to SCO or fibrotic tubules. Nevertheless, in principle, survival and development even up to meiosis was found in at least one tubule of one ectopic graft of an adult recipient, indicating that successful graft support is possible but obviously much more difficult with material derived from a mature testis.

**Fully castrated animals, experiment 2: transplantation site and cryopreservation**

Group 3. We analyzed 18 of 20 originally ectopically grafted fragments from the back skin and 16 of 20 orthotopic grafts from the scrotum, respectively (Table 1 and Fig. 3). In the ectopic grafts (Fig. 3, A and B), degenerating spermatocytes (32.1 ± 28.9% of the tubules per section; Table 2) but no further advanced spermatogenic cell stages were found (Table 2 and Fig. 4, A and B). However, the orthotopic grafts in the scrotum exhibited (Fig. 3C) complete spermatogenesis (12.4 ± 12.2% of the tubules; Table 2) in all animals, and some tubules contained morphologically normal spermatooza (Table 2 and Fig. 4, C–F). Such spermatooza were analyzed by smear preparation immediately after explantation and were found to be immotile (Fig. 4G). In the pregraft control tissues, only gonocytes and early spermatogonia localized at the basal membrane were observed. In this group of castrated animals, the average testosterone values remained low over the entire study period, just above the detection limit (Fig. 2A).

Group 4. Fourteen of 20 of the transplanted fresh grafts and none of the cryopreserved grafts survived (Table 1). Tissue fragments could be analyzed from all five animals; they contained tubules with meiotic arrest and spermatocytes as the most advanced germ cell type (23.5 ± 27.3% of the tubules; Table 2). The remaining sample showed arrest at the spermatogonial level, SCO, or fibrotic degeneration. In the pregraft control tissue, only gonocytes and early spermatogonia were found. The testosterone levels remained low, comparable to group 3 (Fig. 2A).

**TABLE 2.** Histological summary of the transplants grouped according to treatment

<table>
<thead>
<tr>
<th>Location</th>
<th>Total no. of tubules per section</th>
<th>Elongating spermatids</th>
<th>Round spermatids</th>
<th>Spermatocytes</th>
<th>Spermatogonia/geronocytes</th>
<th>Sertoli cells</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1 (4 wk), back: fresh grafts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testis juvenile</td>
<td>231</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>100 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Back skin graft</td>
<td>258</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>28.7 ± 30.9</td>
<td>53.4 ± 27.1</td>
<td>15 ± 21.7</td>
</tr>
<tr>
<td>Testis adult</td>
<td>408</td>
<td>94 ± 6.1</td>
<td>4 ± 5.8</td>
<td>1.8 ± 1.7</td>
<td>0 ± 0</td>
<td>0.2 ± 0.4</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Group 2 (adult), back: fresh grafts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testis adult before</td>
<td>337</td>
<td>92.4 ± 8.5</td>
<td>2.6 ± 2.6</td>
<td>4.5 ± 6.5</td>
<td>0.6 ± 1.3</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Back skin graft</td>
<td>196</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.4 ± 1.2</td>
<td>1.7 ± 3.2</td>
<td>11.7 ± 18.1</td>
<td>86 ± 20.1</td>
</tr>
<tr>
<td>Contralateral testis adult</td>
<td>469</td>
<td>89.5 ± 9.9</td>
<td>8.2 ± 8.4</td>
<td>2.2 ± 2.1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td><strong>Group 3 (4 wk), back and scrotum: fresh grafts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testis juvenile</td>
<td>242</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>94.3 ± 10.9</td>
<td>5.7 ± 10.9</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Back skin graft</td>
<td>1027</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>32.1 ± 28.9</td>
<td>18.4 ± 14.2</td>
<td>33.1 ± 26.3</td>
<td>16.4 ± 17.4</td>
</tr>
<tr>
<td>Scrotum graft</td>
<td>842</td>
<td>12.4 ± 12.2</td>
<td>19.3 ± 22.2</td>
<td>17.9 ± 15.2</td>
<td>6.2 ± 8.3</td>
<td>31 ± 31.9</td>
<td>13.2 ± 19</td>
</tr>
<tr>
<td><strong>Group 4 (4 wk), back: fresh &amp; cryopreserved grafts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testis juvenile</td>
<td>206</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>100 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Back skin graft</td>
<td>692</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>23.5 ± 27.3</td>
<td>26.1 ± 19.5</td>
<td>30.5 ± 12.6</td>
<td>19.9 ± 24.2</td>
</tr>
</tbody>
</table>

The columns describe the percentage ± SD of tubules with the most advanced cell type found per transplantation site.
Spermatogenic development was successfully completed up to spermatozoa in orthotopic grafts in group 3 but was arrested at the meiosis stage (spermatocytes) in the ectopically transplanted tissue, indicating that the transplantation site influences maturation of the grafts.

In group 4, the subsequent transplantation of cryopreserved tissue did not affect those transplants that were already freshly engrafted several months before and that developed meiotic arrest, but these stored grafts failed to survive the remaining study period. None of these grafts were found at the time point of analysis. This might be caused either by the increased age of the animals at the time of the second transplantation procedure or by damage of the tissue during cryopreservation.

Comparing the results with regard to the status of castration, evaluation of the ectopic grafts revealed less spermatogenic development in animals with a remaining testis, i.e. in monkeys with normal serum testosterone levels compared with those with very low serum testosterone (Tables 1 and 2).

Spermatogenic development was successfully completed up to spermatozoa in orthotopic grafts in group 3 but was arrested at the meiosis stage (spermatocytes) in the ectopically transplanted tissue, indicating that the transplantation site influences maturation of the grafts.

In group 4, the subsequent transplantation of cryopreserved tissue did not affect those transplants that were already freshly engrafted several months before and that developed meiotic arrest, but these stored grafts failed to survive the remaining study period. None of these grafts were found at the time point of analysis. This might be caused either by the increased age of the animals at the time of the second transplantation procedure or by damage of the tissue during cryopreservation.

Comparing the results with regard to the status of castration, evaluation of the ectopic grafts revealed less spermatogenic development in animals with a remaining testis, i.e. in monkeys with normal serum testosterone levels compared with those with very low serum testosterone (Tables 1 and 2).
FIG. 4. Histology of the grafted testicular tissue and smears of tissue recovered from the scrotum. A, Ectopic graft with tubules containing a lumen (arrow). No cell stages later than spermatocytes were found in this tissue. B, Tubules were mainly fibrotic, and only the tubule walls remained in the inside of the back skin (arrow). Scale bars, 500 μm (A and C) and 200 μm (B and D). C–F, Histology of a scrotal transplant of group 3: C, overview of a transplant dissected in the middle; D, the tubules show different stages of development with some having only spermatocytes and others showing complete spermatogenesis (arrow); E and F, different tubules containing elongating and elongated spermatozoa. Scale bar, 20 μm. G, Three micrographs of marmoset spermatozoa after an orthotopic graft was spread onto a slide. Scale bar, 20 μm.
The area under the curve (AUC) values of the castrated animals (groups 3 and 4) were significantly different from group 1 ($P < 0.001$) (Fig. 2A). The AUC of group 1 (AUC$_{group1}$ = 2606; $P < 0.05$) was higher than the values of the two immature groups (AUC$_{group3} = 338$; AUC$_{group4} = 239$).

In contrast, the BioCG values obtained from the last three samples did not differ significantly among the monkeys (Fig. 2). When BioCG values were correlated with the testosterone levels, the two castrated groups appeared clustered (Fig. 2B). Furthermore, the two hemi-castrated groups differed from the immature animal groups but did not form a cluster. The adult group exhibited higher BioCG values and higher testosterone values, although group 1 had only higher testosterone levels (Fig. 2B).

**Morphometry of the transplants in both experiments**

In accordance with the arrested developmental state of the grafts, tubular and luminal diameters in the transplants were at an intermediate state compared with those of immature controls and adult testes. All differences were statistically significant. Although in immature control tissue, 98.1 ± 6.5% of the tubules contained gonocytes or early spermatogonia as the most advanced germ cell type, complete spermatogenesis up to elongated spermatids was found in 92.4 ± 8.5% of all the tubules from the adult testes (Table 2). The tubular (84.6 ± 1.8 μm) and luminal (48.2 ± 2.0 μm) diameters in the seminiferous tubules of the ectopic grafts obtained from immature treatment groups were similar, whereas the diameters of the seminiferous tubules (34.6 ± 1.7 vs. 29.2 ± 7.4 μm) of the ectopic grafts obtained from the adult treatment group were significantly smaller ($P < 0.001$). The tubular (111.9 ± 2.3 μm) and luminal (61.3 ± 2.0 μm) diameters of the orthotopic grafts from the scrotum in group 3 were significantly ($P < 0.001$) larger than those found in the tubules of ectopic grafts from the back skin but significantly smaller ($P < 0.001$) than those in the adult testes (200.4 ± 2.8 vs. 77.8 ± 2.6 μm). However, the situation in the orthotopic grafts (38.7 ± 5.0 vs. 14.4 μm) obtained from the adult animals of group 2 was comparable to that of the ectopic grafts of the other groups, but diameters of the tubules were significantly smaller than in transplants recovered from the scrotal transplantation sites of the immature groups ($P < 0.001$).

**Discussion**

In a previous approach, we autologously grafted testicular tissue in marmosets into an ectopic position under the back skin of immature, fully castrated animals, and after puberty, we obtained developed tissue but with meiotic arrest (10). Therefore, we designed two experiments to address this maturation arrest of the germ line in autologously grafted material. The first experiment used hemi-castrated monkeys conserving a natural endocrine profile over the experimental period to assess the meaning of hormone milieu, in particular of testosterone. In addition, we asked whether the donor’s age might play a role as observed in other transplantation studies (1). In the second experiment, we performed a study design similar to our previous study (10) in immature castrated animals, but here we analyzed whether the site of transplantation influences the outcome of spermatogenesis and, in addition, whether cryopreserved testis material can be autologously grafted after storage over a longer period.

In the first experiment, the hemi-castrates of group 2 were already adult when they received the grafts derived from their fully matured and functional testis with complete spermatogenesis. The survival and spermatogenic progress of these grafts was marginal; they were almost completely degenerated. In contrast, in immature animals, a higher percentage of explants and a better survival rate of the grafts were observed. Although only on a spermatogonial level, the majority of the grafts contained living germ cells. The developmental age of the grafts at the time point of transplantation might have caused this difference, very likely because of the higher sensitivity to periods of ischemia known to affect mature material more strongly than immature material (33, 34).

The hemi-castrated animals showed normal testosterone values; i.e. in the young animal group, the testosterone values were already elevated before puberty but highly variable. In an earlier study, consecutive blood sampling revealed extreme fluctuations in testosterone concentrations, suggesting an erratic secretion (28, 35). The onset of puberty was ascertained in intact animals by an increase in serum testosterone levels from values of less than 10 nmol/liter to values above 80 nmol/liter (10). Lunn et al. (36) demonstrated higher levels of biologically active testosterone, and Pugeat et al. (37) reported that plasma testosterone–estradiol-binding protein in New World monkeys has a low affinity resulting in high levels of plasma testosterone (20–40 nmol/liter), compared with those in Old World monkeys or humans (3–9 nmol/liter). The difference in binding characteristics may explain the high testosterone values in marmosets. This seems to be a specific feature of marmosets and is in accordance with

2 and Fig. 2A). The area under the curve (AUC) values of the castrated animals (groups 3 and 4) were significantly different from group 1 ($P < 0.001$) (Fig. 2A). The AUC of group 1 (AUC$_{group1}$ = 2606; $P < 0.05$) was higher than the values of the two immature groups (AUC$_{group3} = 338$; AUC$_{group4} = 239$).

In contrast, the BioCG values obtained from the last three samples did not differ significantly among the monkeys (Fig. 2). When BioCG values were correlated with the testosterone levels, the two castrated groups appeared clustered (Fig. 2B). Furthermore, the two hemi-castrated groups differed from the immature animal groups but did not form a cluster. The adult group exhibited higher BioCG values and higher testosterone values, although group 1 had only higher testosterone levels (Fig. 2B).

**Morphometry of the transplants in both experiments**

In accordance with the arrested developmental state of the grafts, tubular and luminal diameters in the transplants were at an intermediate state compared with those of immature controls and adult testes. All differences were statistically significant. Although in immature control tissue, 98.1 ± 6.5% of the tubules contained gonocytes or early spermatogonia as the most advanced germ cell type, complete spermatogenesis up to elongated spermatids was found in 92.4 ± 8.5% of all the tubules from the adult testes (Table 2). The tubular (84.6 ± 1.8 μm) and luminal (48.2 ± 2.0 μm) diameters in the seminiferous tubules of the ectopic grafts obtained from immature treatment groups were similar, whereas the diameters of the seminiferous tubules (34.6 ± 1.7 vs. 29.2 ± 7.4 μm) of the ectopic grafts obtained from the adult treatment group were significantly smaller ($P < 0.001$). The tubular (111.9 ± 2.3 μm) and luminal (61.3 ± 2.0 μm) diameters of the orthotopic grafts from the scrotum in group 3 were significantly ($P < 0.001$) larger than those found in the tubules of ectopic grafts from the back skin but significantly smaller ($P < 0.001$) than those in the adult testes (200.4 ± 2.8 vs. 77.8 ± 2.6 μm). However, the situation in the orthotopic grafts (38.7 ± 5.0 vs. 14.4 μm) obtained from the adult animals of group 2 was comparable to that of the ectopic grafts of the other groups, but diameters of the tubules were significantly smaller than in transplants recovered from the scrotal transplantation sites of the immature groups ($P < 0.001$).

**Discussion**

In a previous approach, we autologously grafted testicular tissue in marmosets into an ectopic position under the back skin of immature, fully castrated animals, and after puberty, we obtained developed tissue but with meiotic arrest (10). Therefore, we designed two experiments to address this maturation arrest of the germ line in autologously grafted material. The first experiment used hemi-castrated monkeys conserving a natural endocrine profile over the experimental period to assess the meaning of hormone milieu, in particular of testosterone. In addition, we asked whether the donor’s age might play a role as observed in other transplantation studies (1). In the second experiment, we performed a study design similar to our previous study (10) in immature castrated animals, but here we analyzed whether the site of transplantation influences the outcome of spermatogenesis and, in addition, whether cryopreserved testis material can be autologously grafted after storage over a longer period.

In the first experiment, the hemi-castrates of group 2 were already adult when they received the grafts derived from their fully matured and functional testis with complete spermatogenesis. The survival and spermatogenic progress of these grafts was marginal; they were almost completely degenerated. In contrast, in immature animals, a higher percentage of explants and a better survival rate of the grafts were observed. Although only on a spermatogonial level, the majority of the grafts contained living germ cells. The developmental age of the grafts at the time point of transplantation might have caused this difference, very likely because of the higher sensitivity to periods of ischemia known to affect mature material more strongly than immature material (33, 34).

The hemi-castrated animals showed normal testosterone values; i.e. in the young animal group, the testosterone values were already elevated before puberty but highly variable. In an earlier study, consecutive blood sampling revealed extreme fluctuations in testosterone concentrations, suggesting an erratic secretion (28, 35). The onset of puberty was ascertained in intact animals by an increase in serum testosterone levels from values of less than 10 nmol/liter to values above 80 nmol/liter (10). Lunn et al. (36) demonstrated higher levels of biologically active testosterone, and Pugeat et al. (37) reported that plasma testosterone–estradiol-binding protein in New World monkeys has a low affinity resulting in high levels of plasma testosterone (20–40 nmol/liter), compared with those in Old World monkeys or humans (3–9 nmol/liter). The difference in binding characteristics may explain the high testosterone values in marmosets. This seems to be a specific feature of marmosets and is in accordance with
Fig. 5. Thermographic and immunohistological images. A, Infrared measurement of the shaved back skin (left side, C01) and scrotum (right side, C01) together with a reference region (C02) of a male marmoset. The arrows indicate the region of interest. The average temperatures within a circle (diameter 5 mm) are given at the top right corner. B and C, BOULE and CDC25A immunostaining of grafts from the back skin (B) and the scrotum (C). The images demonstrate that BOULE (B1 and C1) is expressed at both transplantation sites, whereas CDC25A (B2 and C2) is expressed only in late and postmeiotic germ cells (arrow). Scale bar, 200 μm.
earlier observations in normal prepubertal animals (28). The testosterone levels were at a postpubertal state at the end of the experiment. Nevertheless, spermatogenesis in the surviving grafts did not progress beyond a very early developmental stage, and it has been demonstrated that a blockade of neonatal activation of the pituitary-gonadal axis has only a minor effect on future proliferation of germ cells in marmosets (38). Obviously, normal postpubertal testosterone values did not affect spermatogenic development positively in the grafts. In the semi-castrated animals, the functional contralateral testis may inhibit or at least not support spermatogenesis of the grafts via serum testosterone or other factors (e.g. inhibins). Reliable assays for FSH and inhibin are not available for the marmoset, but in fully castrated animals, FSH must be high compared with semi-castrated marmosets. Treatment of gonadotropin-deficient boys with recombinant FSH leads to testis growth and full spermatogenesis (39). The endogenous elevated FSH values in the fully castrated animals may better support the remaining Sertoli cells to mature and support the adjacent germ cells of the grafts. The orthotopic grafts in the fully castrated animals reach full spermatogenesis, which implies that, independent of the implant position, spermatogenesis is initiated, but the back skin temperature at the ectopic grafts prevents their meiotic maturation. Insufficient FSH levels in the grafts of semi-castrated animals might prevent spermatogenic onset.

In accordance with our previous study (10), in the castrated animals, serum testosterone levels remained at baseline. It is likely that the Leydig cells in the grafts were functional but unable to raise the serum testosterone levels to normal values because of their limited number (10). As in our previous study, we analyzed the Leydig cells of grafts from all four animal groups for their 5α-reductase expression to determine their maturity. The results of this study in all animal groups were similar and confirmed our earlier findings (data not shown). Thus, in the marmoset, high circulating serum testosterone levels are not essential for the orthotopic grafts to complete spermatogenesis. Even local testosterone might be of minor significance for the maturation of marmoset germ cells. When xenologously cografted with hamster tissue, marmoset germ cells were also arrested at spermatogonial level, although the surrounding tubules of rodent origin matured to haploid cells (7).

High gonadotropin (CG) levels in the castrated animals (groups 3 and 4) are not necessary to induce early events of spermatogenesis up to a meiotic state because the hemicastrated animals also had high serum CG. The levels were even higher than observed in other animals at the onset of puberty (28). It might be that the hemicastrated immature animals had a different endocrine profile compared with those castrated during puberty. If so, testosterone feedback could have lowered the CG peaks in these animals. To evaluate this question, our study design would have required an extra animal group of hemicastrated orthotopically transplanted animals. The contralateral testis of these animals would have normal spermatogenesis and relatively normal sex hormone values throughout their lifetime to support the transplants in the scrotum. However, CG alone cannot be responsible for differences in spermatogenic progress because the animals of group 3 were all castrated, and thus, independent of the transplantation site, the grafts in the castrates, which were all found to be fully vascularized, must have had similar systemic gonadotropin levels to support local testosterone production for maintenance and stimulation of germline differentiation. Nevertheless, the ectopically transplanted tissue exposed to high gonadotropin levels were also found to be arrested at meiosis.

Testicular tissue obtained from immature monkeys can complete spermatogenesis when grafted autologously into the scrotal position. In contrast, testicular fragments transplanted ectopically under the back skin were arrested at the meiotic level. Testicular maturation of the orthotopically positioned grafts also occurred when ectopic grafts in the same individuals failed to develop beyond meiosis (4, 7, 10, 40). This observation suggested that local factors influence developmental progress at the different transplantation sites that either support the grafts (scrotal) or block their full maturation (back skin).

When we compared the ectopic transplantation site under the back skin with the orthotopic scrotal position by thermographic imaging of three normal male animals, we found a substantial difference of almost 5°C. The temperature of the scrotal skin has been demonstrated to also be a suitable marker for testicular temperature (41). Furthermore, our finding is consistent with results in men determining the scrotal temperature to be around 32–35°C (reviewed in Ref. 42).

The higher temperature at the ectopic transplantation site on the back of the animals may contribute to the developmental arrest of germ cells. Such impact on germ cell maturation has been reported in other species and was also used as an experimental method for male contraception (43, 44). In early contraception attempts for men, many different techniques were performed to increase testicular temperature (reviewed in Ref. 42). In an efficacy study, even a mild temperature increase of 2°C resulted in infertility in all volunteering subjects (45). In bulls, a clear correlation was demonstrated, showing that the higher the gradient between body and scrotum temperature, the better the sperm quality (46). Only transplantation into an orthotopic scrotal position resulted in fully matured grafts. The most prominent difference between the scrotum and the back is the local temperature, and thus, this local factor might be a good candidate to explain spermatogenic arrest in the ectopic transplantation sites (10, 25).

Testicular grafting in animal models aims at new routes toward fertility preservation in prepubertal boys threatened by infertility due to disease treatments and destroying the germline. To date, the most advanced methodology for tissue preparation is to cryopreserve testicular biopsies, and in experimental studies, successful transplantation experiments have been reported (11, 21, 47). Therefore, we additionally examined the development of cryopreserved testis tissue to analyze the survival and progression of such material autologously grafted onto the back skin of animals that previously had received fresh testis tissue. Although more than 60% of the fresh grafts could be recovered and some developed up to the premeiotic stage, none of the cryopreserved transplants survived. Because our surgery procedure worked well in the material grafted freshly, we exclude that tissue loss occurred because of a surgical problem or an
infection. Although we could clearly determine the transplantation sites by the surgical scars, neither was tissue found nor were any signs of inflammation seen. This failure may have been caused by an insufficient cryopreservation protocol, although a similar protocol has been successfully used in other experiments (2, 9). Alternatively, the recipients aged between both time points of transplantation. Thus, increased age at the second grafting date, the transplantation of the cryopreserved material, might also have had an adverse effect on the transplanted tissue. Testicular grafting for clinical purposes could be an option but only if the possible risks can be resolved (48).

In summary, we demonstrate for the first time that autologous grafting of nonhuman primate testicular tissues organized similarly to human testes resulted in full spermatogenesis in grafts retransplanted into the scrotum of immature animals. Our findings indicate that orthotopic transplantation and the developmental age of the tissue play most important roles for grafting success. Factors involved might be the temperature as well as the resistance against a period of hypoxia. Factors such as position of the graft, size of the graft, angiogenesis, and adequate cryopreservation protocols of testicular tissue material have to be carefully evaluated further in this nonhuman primate model.

Acknowledgments
We thank O. Damm, J. Salzig, H. Kersebom, and R. Sandhowe for technical assistance, R. Chandolia for veterinarian advice, and S. Ni-eschlag, M.A., for language editing of the manuscript. The authors are indebted to T. Mondritzki of Bayer Healthcare AG in Elberfeld, Ger-

References
2. Schlatt S, Kim SS, Gosen R 2002 Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and het-

Endocrinology, April 2008, 149(4):1736–1747
Luetjens et al.  Spermatogenesis in Grafted Testicular Tissues

2006 Meiosis in autologous ectopic transplants of immature testicular tissue grafted to Callithrix jaccus. Biol Reprod 74:706–713
23. Cortes D, Thorup J, Lindenberg S, Viskelid J 2003 Infertility despite surgery for cryptorchidism in childhood can be classified by patients with normal or elevated follicle-stimulating hormone and identified at orchidopexy. BJU Int 91:670–674
34. Schmidt JA, de Avila JM, Mclean DJ 2006 Grafting period and donor age affect the potential for spermatogenesis in bovine ectopic testis xenografts. Biol Reprod 75:160–166
in the marmoset and their gonadotrophin dependence: a model for investigating susceptibility of the prepubertal human testis to cancer therapy? Hum Reprod 17:1367–1378


Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.