ARTICLE

Exogenous administration of recombinant human FSH does not improve germ cell survival in human prepubertal xenografts

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Abstract In a previous study, meiotic activity was observed in human intratesticular xenografts from peripubertal patients. However, full spermatogenesis could not be established. The present study aimed to evaluate whether the administration of recombinant human FSH could improve the spermatogonial survival and the establishment of full spermatogenesis in intratesticular human xenografts. Human testicular tissue was obtained from six boys (aged 2.5–12.5 years). The testicular biopsy was fragmented and one fragment of 1.5–3.0 mm³ was transplanted to the testis of immunodeficient nude mice. Transplanted mice were assigned to different experimental groups to enable evaluation of the effects of FSH administration and freezing. The structural integrity of the seminiferous tubules, the spermatogonial survival and the presence of differentiated cells were evaluated by histology and immunohistochemistry. Freezing or administration of FSH did not influence tubule integrity and germ cell survival in human xenografts. Meiotic germ cells were observed in the xenografts. More tubules containing only Sertoli cells were observed in frozen–thawed grafts, and more tubules with meiotic cells were present in fresh grafts. There was no clear influence of FSH treatment on meiotic differentiation. Administration of FSH did not improve the establishment of full spermatogenesis after intratesticular tissue grafting.

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KEYWORDS: differentiation, FSH, human, xenografting

http://dx.doi.org/10.1016/j.rbmo.2012.11.013
Introduction

Testicular tissue grafting is currently being developed as a fertility preservation method for boys facing spermatogenic stem cell loss caused by gonadotoxic treatments. This implies the cryopreservation of testicular tissue before starting therapy and transplantation of the tissue after cure. Full spermatogenesis has been reported after grafting testicular tissue of different species to the back skin of nude mice (Abrisghami et al., 2010; Honaramooz et al., 2002, 2004; Oatley et al., 2004; Rathi et al., 2006; Schlatt et al., 2002, 2003; Snedaker et al., 2004; Zeng et al., 2006). The efficiency of human xenografting has been explored by several groups, including the current group. Human testicular tissue has been transplanted to the back skin, scrotum and testes of immunodeﬁcient mice (Geens et al., 2006; Goossens et al., 2008; Sato et al., 2010; Schlatt et al., 2006; Van Saen et al., 2011; Wyns et al., 2008). Limited spermatogenic survival was reported in ectopic human xenografts from both adult and prepubertal tissue. However, severe sclerosis and mostly degenerated tubules were observed in adult xenografts (Geens et al., 2006; Goossens et al., 2008; Schlatt et al., 2006). Differentiation up to pachytene spermatocytes was achieved in testicular tissue transplanted to the scrotum (Wyns et al., 2008) and in ectopic xenografts from neonatal tissue (Sato et al., 2010). This study group recently reported that the testis could also be a suitable transplantation site, since a higher spermatogenital survival was observed compared with ectopic grafting. Meiotic activity was observed in human testicular tissue from post-pubertal boys transplanted inside the testis of immunodeﬁcient mice (Van Saen et al., 2011). However, as far as is known, post-meiotic activity has never been achieved in xenotransplanted human testicular tissue.

Induction and maintenance of spermatogenesis is dependent on the continuous and controlled interaction of several hormones in the hypothalamic–pituitary–testis axis. Sertoli cells have receptors for FSH which is one of the key regulators of spermatogenesis. In response to FSH, Sertoli cells secrete several factors necessary for the progression of spermatogonia into spermatocytes (Sofikitis et al., 2008). It has been reported that FSH regulates spermatogonial survival by the prevention of germ cell apoptosis (Ruwanpura et al., 2008). During the first 2 months after grafting, an initial decrease in spermatogonia has been reported in ectopic bovine grafts (Rathi et al., 2005). In human xenografts, spermatogonial loss was also observed (Van Saen et al., 2011; Wyns et al., 2008).

When testicular tissue is transplanted ectopically, recipient animals are castrated in order to achieve high FSH and LH concentrations, mimicking the situation just before puberty. These high post-castration concentrations are likely responsible for the advancement of testicular maturation as observed in rhesus monkey xenografts. Full spermatogenesis could be obtained in xenografts with a testicular tissue age of 20 months, while rhesus monkeys normally do not reach puberty until about 4 years of age (Honaramooz et al., 2004). Exogenous administration of gonadotrophins was effective in the induction of maturation in infant monkey xenografts since more mature Sertoli cells and more advanced germ cell types were present in treated compared with untreated xenografts (Rathi et al., 2008). FSH has a positive effect on germ cell maturation in cultured rat testis fragments (Meehan et al., 2000) and promotes germ cell meiosis during in-vitro culture of human testicular cells (Tesarik et al., 1998).

Since castration is not possible when intratesticular transplantation has to be performed, FSH and LH concentrations should be increased in a different way. It has been reported that exogenous administration of gonadotrophins promotes post-meiotic differentiation in equine xenografts, while pachytene spermatocytes were the most advanced germ cell type present in untreated grafts (Rathi et al., 2006).

The aim of this study was to evaluate whether exogenous administration of recombinant human FSH could have a positive influence on the outcome of intratesticular human xenografts. It was postulated that: (i) FSH could prevent germ cell loss in the xenografts; and (ii) FSH could induce accelerated maturation and stimulate post-meiotic differentiation in prepubertal xenografts.

Materials and methods

Donor tissue

Human testicular tissue was obtained from six patients, aged 2.5–12.5 years old. All patients had to undergo testicular biopsy before receiving bone marrow transplantation. An overview of indications for testicular freezing and the patients’ treatment history is given in Table 1. After obtaining written informed consent from the boys’ parents, testicular tissue was frozen using a non-controlled slow-freezing protocol with dimethylsulphoxide and sucrose as cryoprotectants, as already described by Van Saen et al. (2011). One fragment was stored in hydrosafe fixative (an alcohol-based, formol-free fixative, R10 S7-16-60; Labonord, Rekkem, Belgium) for reference histology and immunohistochemistry.

Male GFP F1 hybrid mice (n = 11) from 5–7 days old were used as control donors. In previous experiments, allogeneic intratesticular grafting resulted in full spermatogenesis in mouse grafts (Van Saen et al., 2009). The donors were bred in the animal centre of the Vrije Universiteit Brussel according to the following breeding model: male C57Bl inbred (Iffa Credo, Brussel, Belgium) × female SV129 inbred GFP” (gift from the Whitehead institute for biomedical research, Cambridge, MA). In the latter strain, GFP is under the control of the β-actin promoter and is expressed in all cell types.

The experiments were approved by both the internal review board of the UZ Brussel (F.W.O.2004/17D, 29 April 2004) and the Animal Care and Use Committee of the Vrije Universiteit Brussel (08-395-1, 29 September 2008).

Surgical procedure and study design

Transplantation was performed by grafting one human testicular tissue fragment of 1.5–3.0 mm3 in one testis, while in the contralateral testis a mouse tissue fragment was transplanted. Human testicular tissue (fresh or frozen—thawed) from six patients was transplanted to the testis of 4–6-week-old Swiss nu/nu mice (n = 31; Charles River Laboratories, Brussels, Belgium), as has been described by
Van Saen et al. (2011). Fresh and frozen—thawed testicular tissue fragments of each donor (n = 6) were transplanted to several mice. An overview of the number of mice transplanted per patient in each treatment group is given in Table 1. Because of the scarcity of the tissue, only frozen–thawed tissue was transplanted from the 3.5-year-old boy. From patients 2 and 3, two mice, of which one was treated with FSH, received human tissue in both testes.

Some of the transplanted mice from the fresh as well as from the frozen—thawed group were injected subcutaneously with 16 IU recombinant human FSH (Gonal-f 900 IU; Serono, Overijse, Belgium). The first injection was given 1 day before transplantation. After transplantation, injections were given three times a week until the mice were killed.

### Histology

Mice were killed by cervical dislocation 9–12 months after transplantation. The testes were collected, decapsulated and fixed in hydrosafe fixative for at least 1 h. After embedding in paraffin, the whole testis was cut in 5-μm serial sections. Every 10 slides, haematoxylin-eosin (HE) staining was performed to localize the human testicular graft in the mouse testis. Mouse and human tubules were clearly distinguishable on HE-stained slides by morphology. The most advanced germ cell type in all human tubules was also recorded on HE-stained slides. Spermatogonia were recognized as large rounded cells with a large round nucleus and abundant cytoplasm. Meiotic germ cells were identified by the presence of diffuse nuclear staining.

### Immunohistochemistry

Consecutive slides were used for immunohistochemistry with MAGE-A4 (1:200) as a human-specific spermatogonial marker, vimentin (1:200) as a human-specific Sertoli cell marker and Boll (1:400) as a meiotic marker. Green fluorescent protein (GFP) antibody (1:100) was used to detect and evaluate the presence of full spermatogenesis in the GFP murine control tissue. Immunostaining was performed as described before (Van Saen et al., 2011). Tubules with the presence of elongated spermatids were considered as tubules with full spermatogenesis.

Structural integrity was determined on vimentin-stained slides. Tubules with well-preserved spermatagonia—Sertoli cell adhesion and attachment of spermatogonia and Sertoli cells to the basal lamina were scored as intact tubules. Germ cell survival was evaluated by MAGE-A4 staining. Only intact tubules were evaluated for the presence of germ cells. The percentage of MAGE-A4+ tubules was determined, as well as the number of MAGE-A4+ cells per tubule. Human tubules that did not contain MAGE-A4+ cells were clearly distinguished from mouse tubules by morphology. The germ cell survival was calculated by dividing the number of MAGE-A4+ cells per tubule after transplantation or after freezing by the number of MAGE-A4+ cells in fresh donor tissue.

### Statistics

Data are presented as numbers (percentages), mean ± standard deviation or mean (standard error, SE).

A two-way between-groups analysis of variance (ANOVA) was conducted to explore the impact of fresh versus frozen—thawed graft status, and FSH versus no FSH stimulation on, in turn, the percentage of normal tubules and germ cell survival in human xenografts and percentage spermatogenesis in mouse grafts. Full factorial models were fitted. Multiple regression models were fitted to quantify the independent effects of fresh versus frozen—thawed graft status, and FSH versus no FSH stimulation on each outcome of interest, i.e. in turn, percentage of normal tubules and germ cell survival in human xenografts and percentage spermatogenesis in mouse grafts. Distributions among groups of Sertoli cell only (SCO) tubules, tubules with spermatogonia and meiotic cells as the most advanced cell types were assessed by the chi-squared test. The alpha-level was set at 0.05.

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**Table 1** Overview of patient history.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Disease</th>
<th>Treatment before biopsy</th>
<th>Transplanted mice (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chemotherapy (mg/m²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Other</td>
<td>Fresh No FSH FSH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Frozen—thawed No FSH FSH</td>
</tr>
<tr>
<td>1</td>
<td>3.5</td>
<td>Neuroblastoma stage IV</td>
<td>Carboplatin 1500 Etoposide (VP 16) 1400 Vincristine 12 Cisplatin 280 Cyclophosphamid 4000</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>12.5</td>
<td>β-Thalassaemia major</td>
<td>Hydroxyurea</td>
<td>Serum ferritin</td>
</tr>
<tr>
<td>3</td>
<td>12.0</td>
<td>Chronic granulomatous disease</td>
<td>None</td>
<td>Interferon γ1b</td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>Chronic granulomatous disease</td>
<td>None</td>
<td>Sporanox Interferon γ1b</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>Sickle cell anaemia</td>
<td>Hydroxyurea</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>5.5</td>
<td>Sickle cell anaemia</td>
<td>Hydroxyurea</td>
<td>None</td>
</tr>
</tbody>
</table>

*One mouse received a human testicular xenograft in both testes.*
Data were analysed using SPSS Statistics version 20 (IBM Corporation).

Results

Recovery

In the fresh non-treated group, one mouse died and nine out of 10 (90.0%) remaining human xenografts were recovered. Data for germ cell survival and tubule integrity from one xenograft are lacking due to a problem with the staining. In the frozen–thawed non-treated group, six out of eight (75.0%) human xenografts were recovered, of which two grafts were degenerated. All non-treated control mouse grafts were recovered in the fresh (6/6) as well as in the frozen–thawed (4/4) group.

In the fresh FSH-treated group, one mouse died and seven out of the eight (87.5%) remaining xenografts were recovered. Six out of seven (85.7%) xenografts were recovered in the frozen–thawed FSH-treated group, of which one xenograft was degenerated. All treated control mouse grafts were recovered in the fresh (8/8) as well as in the frozen–thawed (7/7) group, although one mouse graft showed sclerosis in the frozen–thawed group.

Characterization of mouse grafts

Mouse grafts were characterized by GFP staining (Figure 1A, B). All GFP⁺ tubules in 20 cross-sections were evaluated for the presence of elongated spermatids. Full spermatogenesis was observed in all mouse grafts, except for the one which showed sclerosis in the frozen–thawed FSH-treated group. In the fresh group, 64.1 ± 7.0% of the tubules showed full spermatogenesis in the non-treated group, while 53.3 ± 13.7% of the tubules contained elongated spermatids in the FSH-treated group. In the frozen–thawed group, 61.4 ± 12.5% of the tubules contained elongated spermatids in the non-treated group and 56.3 ± 7.3% in the FSH-treated group (Figure 1C). The two-way between-groups ANOVA showed that there was no statistically significant effect on the percentage of spermatogenesis for fresh versus frozen–thawed or FSH versus no FSH graft status.

Structural integrity of the human intratesticular xenografts

Donor testicular tissue was characterized by vimentin for tubule integrity. Tubule integrity in the donor tissue before transplantation ranged from 64.7% to 95.3% intact tubules.
2.1–11.1 MAGE-A4+ cells per tubule. The percentage of MAGE-A4+ tubules in the fresh tissue, 240–535 tubules in six cross-sections were evaluated. The patient-specific results of the xenografts in each treatment group can be found in Table 3. Figure 3A shows the mean structural integrity after transplantation in the untreated and FSH-treated groups. In the fresh xenografts, 59.9 ± 25.3% of the tubules in the non-treated grafts were scored intact, while 56.0 ± 16.9% of the tubules were intact in the FSH-treated group. In the frozen–thawed group, 59.0 ± 23.9% were intact in the non-treated group and 72.3 ± 16.6% in the FSH-treated group. The two-way between-groups ANOVA showed that there was no statistically significant effect on structural integrity for fresh versus frozen–thawed or FSH versus no FSH graft status. The estimated marginal means (SE) were 57.9% (5.5%) and 65.7% (7.1%) for fresh versus frozen–thawed, respectively, and 59.4% (6.5%) and 64.2% (6.2%) for FSH versus no FSH (Figure 3B). In the multiple regression model with structural integrity as the outcome of interest, FSH versus no FSH, fresh versus frozen–thawed graft status explained only 5% of the variance (adjusted $R^2 = 0.047$).

### Table 2: Tubule integrity, presence of germ cells and differentiation in fresh donor tissue before transplantation.

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of T</th>
<th>Intact T (%)</th>
<th>No. of T</th>
<th>MAGE+ T (%)</th>
<th>No. of MAGE+ cells/T</th>
<th>Differentiation (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>212</td>
<td>95.3</td>
<td>250</td>
<td>80.0</td>
<td>2.2</td>
<td>20.0 80.0 0.0</td>
</tr>
<tr>
<td>2</td>
<td>306</td>
<td>64.7</td>
<td>240</td>
<td>96.7</td>
<td>11.1</td>
<td>3.3   28.0 68.7</td>
</tr>
<tr>
<td>3</td>
<td>269</td>
<td>75.5</td>
<td>304</td>
<td>90.8</td>
<td>10.4</td>
<td>3.3   40.0 56.7</td>
</tr>
<tr>
<td>4</td>
<td>208</td>
<td>69.2</td>
<td>260</td>
<td>72.3</td>
<td>2.8</td>
<td>27.7 72.3 0.0</td>
</tr>
<tr>
<td>5</td>
<td>97</td>
<td>82.5</td>
<td>539</td>
<td>62.1</td>
<td>2.3</td>
<td>37.9 62.1 0.0</td>
</tr>
<tr>
<td>6</td>
<td>103</td>
<td>90.3</td>
<td>535</td>
<td>49.2</td>
<td>2.1</td>
<td>50.8 49.2 0.0</td>
</tr>
</tbody>
</table>

SCO = Sertoli cell only; SPGIA = spermatogonia; T = tubules.

<sup>a</sup>The evaluated tubule number for germ cell survival was also used to calculate the distribution of tubules with Sertoli cells, spermatogonia or meiotic cells as the most advanced germ cell type.

The human testicular tissue graft was located using vimentin staining (Figure 2) and the integrity of the tissue was determined. The patient-specific results of the human xenografts in each treatment group can be found in Table 3. Figure 3A shows the mean structural integrity after transplantation in the untreated and FSH-treated groups. In the fresh xenografts, 59.9 ± 25.3% of the tubules in the non-treated grafts were scored intact, while 56.0 ± 16.9% of the tubules were intact in the FSH-treated group. In the frozen–thawed group, 59.0 ± 23.9% were intact in the non-treated group and 72.3 ± 16.6% in the FSH-treated group. The two-way between-groups ANOVA showed that there was no statistically significant effect on structural integrity for fresh versus frozen–thawed or FSH versus no FSH graft status. The estimated marginal means (SE) were 57.9% (5.5%) and 65.7% (7.1%) for fresh versus frozen–thawed, respectively, and 59.4% (6.5%) and 64.2% (6.2%) for FSH versus no FSH (Figure 3B). In the multiple regression model with structural integrity as the outcome of interest, FSH versus no FSH, fresh versus frozen–thawed graft status explained only 5% of the variance (adjusted $R^2 = 0.108$).

**Germ cell survival in the human intratesticular xenografts**

Germ cell survival was determined by MAGE-A4 staining (Figure 4 and Table 2). The percentage of seminiferous tubules containing MAGE-A4<sup>+</sup> cells was determined, as well as the number of MAGE-A4<sup>+</sup> cells per tubule. In the donor tissue, 240–535 tubules in six cross-sections were evaluated. The percentage of MAGE-A4<sup>+</sup> tubules in the fresh biopsy ranged from 49.2% to 96.7%, corresponding with 2.1–11.1 MAGE-A4<sup>+</sup> cells per tubule.

All cross-sections through the whole graft were analysed. The patient-specific results of the xenografts in each treatment group can be found in Table 3. Figure 3C shows the mean germ cell survival in the non-treated and FSH-treated groups. Germ cell survival was 93.7 ± 62.3% in the fresh untreated group and 53.4 ± 40.5% in the fresh FSH-treated group. In the frozen–thawed group, a germ cell survival of 83.9 ± 59.5% was observed in the untreated group, while this was 39.2 ± 32.7% in the FSH-treated group. Although there was a tendency for a lower germ cell survival in the FSH-treated group, this did not reach statistical difference in the two-way between-groups ANOVA. The estimated marginal means (SE) were 73.6% (13.2%) and 61.5% (17.1%) for fresh versus frozen–thawed, respectively, and 46.3% (14.9%) and 88.8% (15.6%) for FSH versus no FSH, respectively (Figure 3D).

In the multiple regression model with germ cell survival as the outcome of interest, FSH versus no FSH, fresh versus frozen–thawed graft status explained 11% of the variance (adjusted $R^2 = 0.108$).

**Meiotic activity in intratesticular xenografts**

Human donor tissue and all human grafts were analysed to record the most advanced germ cell stage. The most advanced germ cell stage present was determined morphologically on HE-stained slides. Patients could be divided into two groups based on the presence of advanced germ cells. In the four youngest patients (2.5–5.5 years old), spermatogonia were the most advanced germ cells present at the time of grafting (Figures 5A and 6A). However, in the two older patients (12.0 and 12.5 years old), meiotic cells were present at the time of grafting (Figures 5F and 6A).

In a previous study, this study group reported meiotic activity in intratesticular grafts from older boys, but not in grafts from younger ones (Van Saen et al., 2011). In the current study, differentiation up to pachytene spermatocytes was observed in grafts from older patients as well as in grafts from younger boys. No differentiation was found in the xenografts from patient 1. Differentiation was found in the untreated grafts as well as in the FSH-treated grafts (Figure 5 and Table 3).

The presence of meiotic activity was evaluated by immunohistochemistry using the meiotic marker Boll. Expression of Boll was only found in the untreated testicular graft from the 12.5-year-old patient. Boll expression was observed in the cytoplasm of spermatocytes but also in the nuclei of spermatogonia (Figure 5J). No expression of Boll could be observed in all the other grafts, although morphologically similar cell types were present (Figure 5E).

The chi-squared test revealed a statistical difference in the distribution among groups of SCO tubules, tubules with spermatogonia and meiotic cells as the most advanced cell types ($P < 0.001$). A significant difference was also found between fresh versus frozen–thawed and FSH versus no FSH ($P < 0.001$). More SCO tubules were observed in fro-
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Figure 2  Tubule integrity of intratesticular xenografts determined by vimentin staining. (A) Human adult testis tissue as positive staining control. (B) Mouse adult testis tissue as negative control. (C–J) Localization of the human graft inside the mouse testis in untreated (C, D and G, H) and FSH-treated (E, F and I, J) groups: (C–F) frozen–thawed intratesticular grafts from a 5.5-year-old donor; (G–J) fresh intratesticular grafts from a 12.5-year-old patient. Asterisks indicate unstained mouse tubules. D, F, H, J are at a larger magnification of some tubules shown in C, E, G, I. Black triangles indicate tubules scored as intact (tubules with well-preserved spermatogonia–Sertoli cell adhesion and attachment of spermatogonia and Sertoli cells to the basal lamina); white triangles indicate tubules scored as not intact (tubules with disturbed intercellular adhesion and/or attachment of spermatogonia and Sertoli cells to the basal lamina). Bars = 100 µm (A, B, D, F, H, J), 1000 µm (C), 600 µm (E), 300 µm (G) and 500 µm (I).

Zen–thawed grafts. Meiotic cells were observed in all treatment groups and no clear influence of FSH treatment was observed. In the fresh group, more tubules containing meiotic cells were observed in the non-treated group, while the
### Table 3  Overview of results of human xenografts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Patient</th>
<th>No FSH</th>
<th>FSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>1</td>
<td>No transplantation performed</td>
<td>Not performed</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35.0</td>
<td>444</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>12</td>
<td>59.0</td>
<td>29.7</td>
</tr>
</tbody>
</table>

**GS = germ cell survival; MC = meiotic cells; SCO = sertoli cell only; SPGIA = spermatogonia; T = tubules.**

*The evaluated tubule number for germ cell survival was also used to calculate the distribution of tubules with Sertoli cells, spermatogonia or meiotic cells as the most advanced germ cell type.*
opposite was true for the frozen–thawed group. When groups were compared one by one, a statistical difference was found between each group ($P < 0.001$; Figure 6B).

Discussion

This study evaluated the effect of recombinant human FSH on the tubule integrity, germ cell survival and differentiation capacity in human intratesticular grafts. This effect was studied in both fresh and frozen–thawed grafts.

Although a lower germ cell survival was observed in the FSH-treated grafts compared with the untreated grafts, this difference was not statistically significant. The fact that a tendency towards lower germ cell survival was observed in the FSH-treated grafts was surprising. FSH is known to regulate glial cell line-derived neurotrophic factor secreted by Sertoli cells (Ding et al., 2011). Glial cell line-derived neurotrophic factor is reported to be an essential growth factor for the self-renewal of spermatogonial stem cells (Kubota et al., 2004). Efficient stimulation of the Sertoli cells by FSH in the xenograft is thus important for spermatogonial stem cell proliferation. However, exogenous administration of FSH did not increase germ cell survival in the human xenografts. However, it should also be noted that a high variability was observed between grafts from different patients. This is inherent to the limited availability of human prepubertal testicular tissue for research.

Patients of different ages and with different treatment history before the biopsy was taken were used in this study. This could have influenced the results.

Some of the patients in this study had already received chemotherapy or some kind of other treatment before the testicular biopsy was taken. The possibility that these previous treatments could have affected the ability of the testicular tissue to respond to FSH should be considered. Although germ cells were present in all biopsies, the functionality of spermatogonial stem cells could be affected by the treatment. Additionally, three patients were treated with hydroxyurea before the testicular biopsy was taken. Long-term treatment with hydroxyurea can affect fertility (Berthaut et al., 2008). Oligo- and azoospermia was reported in four patients who received hydroxyurea starting from childhood to adulthood (Lukusa and Vermylen, 2008). However, also interferon $\gamma$ could affect testicular function (Bussiere et al., 1996), which means that all patients in this study received some kind of treatment with a possible adverse effect on fertility. This study was performed albeit the fact that the functionality of the germ cells could be affected. Testicular tissue from patients who had chosen to preserve testicular tissue before starting conditioning therapy for bone marrow transplantation was used. It will be these patients who will come back to the fertility centre within a few years with the request for transplantation. Since most of the patients did receive some kind of treat-
ment before banking, possibly damaged tissue will also be used in a clinical application.

This study postulated that FSH stimulation could induce post-meiotic differentiation in the human intratesticular xenografts as observed in equine and monkey xenografts (Rathi et al., 2006, 2008). In this study, differentiation was observed in grafts from both older and young patients. However, treatment with exogenous FSH did not stimulate meiotic differentiation. It seems that endogenous FSH was sufficient to stimulate the initiation of differentiation, but post-meiotic differentiation was not observed in the human intratesticular xenografts.

The grafting site can influence the graft outcome. Full spermatogenesis was also not achieved in ectopic xenografts from marmosets (Schlatt et al., 2002) and autologous ectopic grafts (Wistuba et al., 2006). However, full spermatogenesis was achieved in orthotopic marmoset transplants, suggesting that hyperthermia was responsible for meiotic arrest in ectopic xenografts (Luetjens et al., 2008). The absence of post-meiotic differentiation in the human xenografts in this study cannot be explained by hyperthermia since grafting was performed in the testis. The xenograft model could influence the possibility of full differentiation. The murine host environment could not be suitable to allow full maturation of human testicular tissue, while full spermatogenesis occurred in the murine grafts. This limits the value of xenografting for the assessment of differentiation in human xenografts. In the literature, full human spermatogenesis in ectopic xenografts has neither been achieved when the mouse was used as a host (Geens et al., 2006; Goossens et al., 2008; Sato et al., 2010; Schlatt et al., 2006). Xenografted cryopreserved testicular rhesus monkey tissue resulted in SCO tubules, while full spermatogenesis was observed in autologous scrotal grafts (Jahnukainen et al., 2012). In a clinical application, autologous transplantation will be performed. Transplanting the testicular tissue in its natural host environment might create the ideal conditions to allow full differentiation.

In the study by Rathi et al. (2008), stimulation with exogenous gonadotrophins induced Sertoli cell maturation, as shown by the expression of the Sertoli cell maturation markers anti-Müllerian hormone and androgen receptor. Meiotic arrest could be associated with incomplete maturation of Sertoli cells in the human xenografts. The persistence of anti-Müllerian hormone, as observed in a previous study by this group (Van Saen et al., 2011), illustrated that Sertoli cells do not reach full maturity in the xenografts. Incomplete maturation of Sertoli cells was also observed in infant monkey and fetal bovine xenografts in which meiotic arrest was also observed (Rathi et al., 2008; Rodriguez-Sosa et al., 2011).

Testosterone is critical for the completion of meiosis (Ruwanpura et al., 2010). In a study by Rathi et al. (2008), monkey xenografts were treated with pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (HCG) as alternatives for FSH and LH. Grafts were either treated with HCG alone or with a combination of HCG and PMSG. However, no difference in graft development was observed between grafts treated with HCG alone or in combination with PMSG. Therefore, the effect on graft development was attributed to increased production of testosterone by the Leydig cells in the graft. In the current study, testosterone is produced by the mouse Leydig cells, but whether the Leydig cells in the graft are producing testosterone remains unknown. In ectopic grafts, it has already been shown that Leydig cells are able to produce testoster-

Figure 4  Germ cell survival of intratesticular xenografts determined by MAGE-A4 staining. (A) Human adult testis tissue as positive staining control. (B) Mouse adult testis tissue as negative control. (C, D) Donor tissue before transplantation from a 5.5-year-old donor (C) and a 12.5-year-old donor (D). (E–H) Frozen–thawed intratesticular grafts from a 5.5-year-old donor. (I–L) Fresh intratesticular grafts from a 12.5-year-old donor. (E, F and I, J) Untreated group. (G, H and K, J) FSH-treated group.
Figure 5  Differentiation in intratesticular xenografts determined by HE staining. The most advanced germ cell stage present was determined morphologically. (A, F) HE staining of pregrafted tissue from a 5.5-year-old donor (A) and a 12.5-year-old donor (F). (B, C) Differentiation in frozen—thawed intratesticular grafts from a 5.5-year-old patient in the untreated (B) and FSH-treated (C) groups. (D, E) The presence of meiotic cells was confirmed by the meiotic marker Boll in a 5.5-year-old donor: Boll staining was observed in nuclei of spermatogonia in pregrafted tissue (D); no positive Boll staining was observed in frozen FSH-treated graft (E). (G, H) Differentiation in fresh intratesticular grafts from a 12.5-year-old patient in the untreated (G) and the FSH-treated (H) groups. (I, J) Boll staining was observed in a 12.5-year-old donor in the cytoplasm of spermatocytes in pregrafted tissue (I) and in the cytoplasm of spermatocytes and also in the nucleus of spermatogonia in fresh, untreated graft (J). Arrowheads indicate spermatogonia; arrows indicate spermatocytes. Bars = 100 μm.
Spermatogonia

Meiotic cells

**Distribution of the presence of different germ cell types in human intratesticular grafts in combined treatment groups:** *P* < 0.001; *P* < 0.001. SCO = tubules containing Sertoli cells only.

Figure 6  Distribution of germ cell types in intratesticular xenografts. Tubules were categorized based on the most advanced germ cell type present. (A) Distribution of the presence of different germ cell types in donor testicular tissue at different ages. (B) Distribution of the presence of different germ cell types in human intratesticular grafts in combined treatment groups: *P* < 0.001; *P* = 0.001.

There is a continuing debate whether FSH and testosterone alone are necessary to restore and maintain spermatogenesis or whether testosterone alone is sufficient. The effect of administration of FSH on the restoration of spermatogenesis was evaluated in hpg mice. Testosterone alone could initiate spermatogenesis, while FSH alone increased the numbers of spermatogonia and spermatocytes, but had no effect on post-meiotic differentiation (Singh and Handelsman, 1996).

In one xenograft, the presence of meiotic activity could be confirmed by staining with the meiotic marker Boll, also known as BOULE. Boll is expressed in the cytoplasm of spermatocytes and proceeds through meiosis until early spermatids (Xu et al., 2001). Boll is required for efficient translation of twin mRNA, which is in turn required for the G2/M transition during meiosis (Maines and Wasserman, 1999). Although in the other xenografts the observed cells showed the morphological characteristics of differentiated cells, they did not stain positive for the meiotic marker. Probably, the spermatocytes observed in the grafts failed to initiate meiosis correctly. Lack of Boll protein was reported in infertile men with meiotic arrest, although spermatocytes were present in all testicular biopsies evaluated.

No mutations or polymorphisms were found in these infertile patients, indicating that an upstream regulatory factor is either nonfunctional or not expressed. Currently, these factors are unknown (Luetsjens et al., 2004). If these factors are not conserved between mouse and human, this could explain why meiosis is not established in human xenografts. In the graft in which Boll staining was observed, it was present in the cytoplasm of the differentiated cells, but also in the nucleus of spermatogonia. It has been reported in Drosophila that Boll localizes premeiotically to a perinuclear region and translocates to the cytoplasm at the onset of meiosis (Cheng et al., 1998). The fact that Boll staining is absent in the other grafts, also in the nucleus, suggests that meiosis does not occur properly in these grafts.

If testicular grafting is to be used as a strategy for fertility preservation, it is important to ensure that the freezing process does not affect the functionality of the tissue. In this study, testicular tissue was cryopreserved using uncontrolled slow freezing. No difference was observed between fresh and frozen–thawed grafts for germ cell survival. However, more SCO tubules were observed in the frozen–thawed grafts. This indicates that the freezing procedure might impair the functionality of the tissue. A previous study by this study group did not observe any difference in graft outcome between fresh and frozen–thawed intratesticular grafting of mouse testis tissue (Van Saen et al., 2009). The uncontrolled slow-freeze-protocol used in the current study to freeze human testicular tissue was similar to the freezing protocol used for mouse testicular tissue. This protocol was found to be efficient in preserving the integrity and functionality of mouse testicular tissue (Baert et al., 2012) and has been published for freezing human testicular tissue (Van Saen et al., 2011). This illustrates that, next to cell survival, the evaluation of the functionality is important when freezing protocols are optimized.

In conclusion, administration of FSH did not improve germ cell survival nor did it stimulate differentiation in human xenografts. More research is needed to enable post-meiotic differentiation in human xenografts.

Acknowledgements

The authors would like to thank Mr Fabian Van Haelst and Mr Pierre Hilven for their technical assistance. They are also thankful to Dr G Spagnoli for providing the MAGE-A4 antibody and to Serono for providing the recombinant human FSH. This study was supported by Grants from the Vrije Universiteit Brussel (OZR and Methusalem), the UZ Brussel (Research Foundation Willy Gepts) and the Scientific Research Foundation-Flanders (FWO). Ellen Goossens is a post-doctoral fellow of the Research Foundation-Flanders (FWO).

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 10 September 2012; refereed 19 November 2012; accepted 20 November 2012.