Xenotransplantation of cryopreserved human ovarian tissue into murine back muscle

R. Soleimani¹*, E. Heytens¹, R. Van den Broecke², I. Rottiers³, M. Dhont¹, C.A. Cuvelier³,†, and P. De Sutter¹,†

¹Department of Reproductive Medicine, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium ²Gynecological Oncology, Department of Obstetrics and Gynecology, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium ³Department of Pathology, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium

*Correspondence address. Fax: +32-9-332-49-72; E-mail: reza.soleimani@ugent.be; rezasoleimani@yahoo.com

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BACKGROUND: Ovarian tissue (OT) cryopreservation and transplantation are options for fertility preservation in young female cancer patients.

METHODS: We investigated xenotransplantation of human OT into back muscle (B) of severe combined immunodeficiency mice. OT follicle content was evaluated by stereomicroscopy and pre-transplantation. Xenograft survival, follicular development (with/without FSH administration), apoptosis and vascularization were compared in B- versus K-site (under the kidney capsule) several times after grafting using histology, immunohistochemistry and magnetic resonance imaging. In vitro maturation (IVM) was also performed.

RESULTS: Anastomoses which developed from existing human and invading murine vessels were seen in OT at both sites, but angiogenesis was more prominent at the B- than K-site (P < 0.001). Vascularization and follicle size were correlated in the B-group (Spearman’s coefficient 0.73; P < 0.001). FSH increased early (8 days) micro-vessel formation in B but not in K grafts (P < 0.0001, versus no FSH). B-site grafts showed a better histological morphology and survival (P = 0.0084), formation of larger antral follicles (P = 0.005), more metaphase-II (MII) oocytes, growing follicles (P = 0.028) and slightly fewer apoptotic follicles than K grafts. One MII oocyte from B underwent IVM and reached MII stage next day.

CONCLUSIONS: To our knowledge, this is the first report of MII and IVM–MII oocytes obtained from B xenografts. We report the largest oval-shaped antral follicles containing an MII oocyte obtained after OT xenotransplantation to date. Xenografting in the mouse B should be further explored as a method for human OT transplantation.

Key words: xenotransplantation / ovary cryopreservation / human ovary / muscle / metaphase II oocyte

Introduction

Primary ovarian insufficiency (POI) is one of the side effects of cancer therapy in pediatric and young female oncology patients. POI was previously defined as premature ovarian failure (POF) by many authors (Van den Broecke et al., 2001; Chemaitilly et al., 2006; Sklar et al., 2006). The term POI was first used by Albright et al. (1942) and recently reintroduced by Welt (2008) and Nelson (2009). POI is a result of depletion of the follicular content of the ovaries which leads to the loss of fertility (Nelson et al., 1994; Falcone and Bedaiwy, 2005). Many women with POI have potentially functional follicles remaining in the ovary. The condition is frequently one of intermittent and unpredictable ovarian functions that can persist for decades. It has also been reported that ~5–10% of women diagnosed with POI will conceive and deliver a healthy child (Rebar et al., 1982; van Kasteren and Schoemaker, 1999; Bakalov et al., 2005). However, permanent ovarian dysfunction will be found in some patients.

Cryopreservation of ovarian tissue (OT) for prospective clinical use was introduced many years ago (Gosden, 1992). Growing ovarian follicles are more susceptible to cryo-injury, whereas primordial follicles can better survive freezing (Newton et al., 1996). Therefore, ovarian cortical tissue that contains primordial follicles is mainly cryopreserved for fertility preservation. Important progress has been made by autotransplanting the OT back to the patient heterotopically (Callejo et al.,...
Human ovary xenotransplantation to mouse back muscle

Human OT source, preparation and estimation of the follicular contents

Human OT was obtained from a consenting 22-year-old female-to-male transsexual. Cryopreservation was performed as described previously (Van den Broecke et al., 2001), and the tissue was stored for ~5 years. Rapid thawing was carried out by placing the vials containing cryopreserved human OTs above liquid nitrogen for 30 s and placing them in a water bath at room temperature for ~90 s. Tissue strips were washed immediately, five times for 1 min each, with M-199 medium (Sigma, M-2154, Bornem, Belgium) in 60 mm tissue culture dishes (Falcon; BD 35–3002, VWR, Leuven, Belgium) to remove the cryoprotectant (dimethylsulphoxide, Sigma, D5879). OT strips were cut into small pieces of ~1 mm² with surgical blades (no. 24) and were washed again in M-199 medium for ~5 min. Follicular content of the OT mini strips was evaluated using the method previously described by our group (Soleimani et al., 2006). Briefly, OT strips of ~1 mm² (1 ± 0.12 mm²; mean ± SD) and ~0.5 mm thickness (476 ± 176 µm; mean ± SD) were placed in pre-warmed M-199 medium in a glass-bottom dish (MatTek, Ashland, USA), and follicular content was measured using an Olympus SZ-60 stereomicroscope. OT fragments containing at least five visible follicles were selected and collected in another dish in pre-warmed M-199 medium for transplantation.

Transplantation procedure

OT fragments were collected in a 35 mm tissue culture dish (Falcon; BD 35-3001, VWR) containing 2 ml of pre-warmed MCDB 105 (Sigma, M-6395) × M199 medium (Sigma, M-2154), ratio 1:1. Recipient animals were anesthetized by i.p. injection of Ketamine 75 mg/kg (Ketalar, Ceva, Brussels, Belgium) and medetomidine 1 mg/kg (Domitor, Orion Pharma, Finland) at room temperature. Negative response to toe and tail pinch was used as an indication for anesthesia. Analgesia was provided by i.p. injection of buprenorphine 0.05 mg/kg (Temgesic, Reckitt & Colman, Hull, UK) before surgery. OT fragments were picked randomly and xenotransplanted into the K-site or the B-site of recipient mice. During grafting, both ovaries of the recipient mice were removed by cautery at the top of the uterine horns. Finally, body wall (4–0, Ethicol, Vycril Rapide suture) and skin (Autoclip, BD; Cat no. 427630) incisions were closed. Anti-inflammatory treatment involved s.c. injection of meloxicam 2 mg/kg every 24 h during 3 days after surgery (Boehringer Ingelheim, Brussels, Belgium). All procedures were performed under aseptic conditions.

Transplantation at the K-site

After a dorso-horizontal incision was made in the skin and body wall, the kidney was exteriorized. An OT fragment was selected randomly and inserted under the capsule of the kidney through a small hole made using a fine watchmakers’ forceps (Extra Long Dumont, no. 5s).

Transplantation at the B-site

A small (3 mm) incision was made in the skin on the dorsal midline of the recipient animal. A hole of 3–5 mm deep was made in the B-site, ~10 mm away from the edge of the incision, using fine watchmakers’ forceps (Extra Long Dumont, no. 5s). In order to stimulate angiogenesis, a fine scar was made in the perimysium between the muscles’ mass at the insertion site. The OT fragment was inserted between the perimysium above the scar.

Experiment I: B-site versus K-site

Human OT fragments were selected randomly and without any pre-evaluation for follicular contents, and one OT fragment was xenotransplanted into B-site (left side) and one OT fragment was xenotransplanted...
into K-site (under the capsule of left kidney) (number of animals 22). Animals were injected every second day with 1 IU FSH (Puregon, NV Organon, Oss, the Netherlands) starting 1 week after grafting (Oktay et al., 2000). Graft samples were processed for hematoxylin and eosin (H&E) staining to study graft size and follicular maturation. Follicular development was investigated in both the B- and K-site xenografts at 3, 5 and 7 months after grafting. Some data obtained from this experiment were compared with that from Experiment IV.

Experiment II: early events during first days after grafting

There is limited information available about the events that occur in the days immediately following OT grafting. To ascertain whether the site of grafting can influence the factors affecting OT damage, eight mice were used to study follicular apoptosis, neovascularization and integration of OT in the grafting site during the first 8 days after transplantation. Five OT fragments were transplanted to each site (B and K) in each mouse. Each day, one animal from each group was selected randomly and xenografts with surrounding tissue were collected to study apoptosis [anti-active caspase-3 (AC-3), by immunohistochemical (IHC) staining], neovascularization (anti-human and anti-mouse CD31), graft quality and tissue integration after grafting [H&E staining and electron microscopy (EM)].

Experiment III: the effect of gonadotrophin treatment on graft quality

To study whether the administration of FSH (1 IU) can enhance graft quality by increasing micro-vessel density during the first 8 days of grafting and long term, four OT fragments were grafted at the K-site and another four OT fragments were grafted at the B-site of each animal (n = 8). Two groups of non-gonadotrophin-treated animals served as controls for both the early event (n = 5) and the long-term study (n = 5). Graft survival, tissue integration and neovascularization were studied.

Experiment IV: MRI-assisted xenotransplantation

On the basis of the data obtained from different experiments performed in this study, a final protocol was designed to improve the outcome of human-to-SCID mouse OT xenotransplantation. After thawing four vials of cryopreserved OT fragments, 11 pieces of OT (∼5 × 5 mm²) were obtained and cut into 1 mm² pieces (n = 235). After stereomicroscopic evaluation, 193 OT fragments were of the favorable size and thickness. Follicular contents of the OT fragments were evaluated by stereomicroscope and 60% of these OT fragments contained at least five viable follicles and were used in the study (115/193). To reduce the effect of the dominant growing follicle, only one small OT fragment was xenografted to either the K-site (n = 20) or the B-site (n = 20) in each animal. FSH administration was started from the day of grafting, by injecting 1 IU FSH daily until Day 8 and every second day for long-term grafting. From the fifth month, follicular development in both B- and K-sites in all animals were evaluated by MRI every 2 weeks. Animals with follicles larger than 6 mm were injected every second day with 5 IU FSH for 2 weeks followed by one dose of 10 IU HCG (Pregnyl, NV Organon). Thirty-six hours later, the animals were euthanized by cervical neck dislocation and grafts were collected for histological evaluation. To compare the efficiency of using MRI for pre-stimulation evaluation, a control group of transplanted animals was used after 6 months and final HCG stimulation was performed without MRI visualization. To evaluate vascularization, proliferation and apoptosis, graft samples were processed for human α-SMA (smooth muscle actin), human and mouse-CD31, proliferating cell nuclear antigen (PCNA) and AC-3 IHC. Ultrasonography was also used to monitor the size of the antral follicles in limited numbers of animals. This resulted in the finding of a follicle of 4.6 mm in one animal (see Results).

Competence of oocytes from B-site allografts

In a complementary study, two follicles >6 mm diameter from B-grafts were punctured and oocytes were retrieved. The immature oocyte was subjected to in vitro maturation (IVM) using the protocol described by Vanhoutte et al. (2005).

Magnetic resonance imaging

Animals were anesthetized as explained earlier and placed in the head coil for imaging. MRI was performed on a 3 T magnet (Siemens Magnetom Trio A Tim System with Syngo MR B13). A special sterile box was designed to restrain 16 mice at a time for imaging.

Histology and follicular assessment

Recovered OTs were fixed in 4% buffered paraformaldehyde (Klinipath Geel, Belgium. Ref no. 4078.9020) at 4°C for 2 h, stored in 0.1% precooled paraformaldehyde/phosphate-buffered saline solution at 4°C and processed manually for paraffin embedding. Histomorphological examination was performed after serial sectioning of the entire graft to 4 μm thickness and staining with H&E. Follicles were counted only when the dark-staining nucleolus was seen within the nucleus of the follicles to prevent recounting. Follicles were classified as primordial (oocytes surrounded by one layer of flattened pregranulosa cells), primary (one layer of cuboidal granulosa cells), secondary (with two layers of granulosa cells), pre-antral (with more than two layers of granulosa cells without antrum), antral (with an antral cavity) and metaphase I (MI) and II (MII) (Gougeon, 1986). H&E staining was used to study the quality and the integration of the grafts within the surrounding tissue. Existence of adipose tissue after long-term grafting, necrosis and acellular areas in the grafts were considered to be signs of reduced OT quality.

Histochernistry

Species-specific vascular labeling and study of angiogenesis

Reconstruction of the blood vessels in grafted human OTs was investigated using species-specific anti-human CD31 IHC staining (staining epi-thelial cells of new blood vessels) in 4 μm thick sections to detect the neovascularization. Specific anti-mouse CD31 (platelet endothelial cell adhesion molecule-1—PECAM-1) was used to study the possibility of neo-vascularization of the murine blood vessels in support of grafted human OTs, using the staining method as described previously (Lehr et al., 1997; Gray et al., 2004; Soleimani et al., 2008). Paraffin-embedded mouse colon tissue section was used as a positive control. The species-specificities of mouse anti-human CD31 were checked by IHC analysis of human and mouse control tissues. Paraffin-embedded human tonsil tissue sections were used as a positive control for anti-human CD31. In order to further evaluate the angiogenesis during wound repair and grafting, mature blood vessel density was measured by means of α-SMA regardless of their origin (murine or human), using the Ventana automated staining system (Ventana, Lille, France). It is obvious that blood vessels positive for α-SMA, which are visualized in the first days after grafting, are unlikely to be of murine origin. Therefore, we attributed the staining to existing human blood vessels in the graft. In the long-term grafting experiments, existing mature blood vessels to support the graft blood supply were our concern, regardless of their species origin. Blood vessels were counted at high-power field magnification (×400) in a grid area of 0.15 mm² at five randomly selected positions in 25 different sections in each tissue sample (Kato et al., 2003; Soleimani et al., 2008). This evaluation is based on true visualization of the vessels in the actual field (0.15 mm²). Area quantification was done with Olympus MicroSuite FIVE software (Olympus America). The sections were also lightly
counterstained with hematoxylin before mounting for further evaluations. For negative control, the primary antibody was omitted.

**Proliferating cell nuclear antigen**

The earliest stage of ovarian follicular growth and development is characterized by granulosa cell proliferation and differentiation (Oktay et al., 1995). IHC using PCNA was used to study the proliferative status of growing human ovarian follicles at different stages in grafted OT fragments. Brown staining of the granulosa cells surrounding the follicle was an indication of PCNA expression. The number of PCNA-positive follicles was counted in the whole transplanted OT fragment. Paraffin-embedded human colon cancer tissue sections were used as positive control.

**Apoptosis**

AC-3 antibody (Promega Benelux, Aj Leiden, the Netherlands, Cat. no. G7481) was used to study early apoptosis in the follicles from xenografted OTs. AC-3 enhances apoptotic cell death and proteolytically cleaves and activates other caspases during apoptosis (Hussein et al., 2003; Drakos et al., 2004). Positive staining was indicated as reddish-brown coloring of the cytoplasm/nucleus of the follicles. The number of AC3-positive follicles was counted in the whole transplanted OT fragment. Paraffin-embedded human appendix tissue sections were used as a positive control.

**Transmission EM**

OT fragments were fixed in paraformaldehyde (2%)/glutaraldehyde (2.5%) in 0.1 M sodiumcacodylate buffer at 4°C and stored in the same solution at 4°C until processed. After post-fixation for 5 min in 2% (w/v) osmium tetroxide in the same buffer, the specimen was dehydrated in an ascending alcohol series (ethanol 50, 70, 90, 100%, three times) and propylene oxide, twice. The specimen was covered with propylene oxide–Epon (1:1, 1 h). Finally, the preparation was covered with the polymerization mixture of Epon. The polymerized sediment blocks were used for semi-thin/ultrathin sectioning.

**Statistical analysis**

Levene’s test of homogeneity of variances (α = 0.01) and Kolmogorov–Smirnov test of normality (α = 0.01) were performed to choose the appropriate statistical test. Mann–Whitney U test or Student’s t-test was performed to analyze the mean follicle size, number of follicles, blood vessel counts and PCNA-positive follicles (presented as mean ± SEM). Non-parametric correlation between the mean follicle size, the mean number of blood vessels or the PCNA-positive follicles was analyzed with the Spearman’s test. Numbers of grafts were analyzed with χ² test or the Fisher’s exact test.

When the P-value was <0.05, the difference was considered significant.

**Results**

**Experiment I: B-site versus K-site**

There was no significant difference between the B- and K-site in further follicular development (secondary, pre-antral and antral) 3, 5 or 7 months after grafting (Fig. 1). The number of grafts with at least one antral follicle containing an MII oocyte was not significantly different between the B- and K-site grafting (4/22 versus 2/22, respectively). The number of grafts with more than one follicle was also not significantly different between B and K-site grafts (3/22 versus 1/22, respectively). At the end of the experiment, antral follicles of 7, 10 and 12 mm could be obtained from the B grafts, while the largest follicle from a K-graft was only 3.5 mm under similar conditions (Fig. 2). The average size of antral follicles obtained from B-sites was significantly larger than those from the K-site grafts (4.1 ± 0.58 versus 2.27 ± 0.18 mm; B and K, respectively, P = 0.005).

![Figure 1](image_url) Mean number of human follicles at a specific stage observed in the mouse B-site (open bar) or K-site (solid bar) 3, 5 and 7 months after OT grafting in Experiment I. No significant differences were observed between B- and K-sites (Mann–Whitney U test, P > 0.05).
Experiment II: early events during the first days after grafting

Although we could not detect any evidence of apoptosis in B- or K-sites using anti-AC-3 staining during the first 2 days after grafting, a higher number of grafts containing AC-3-positive follicles were seen at the K-site from days 3 to 7 (14/30 versus 5/30, K- and B-site, respectively, \( P < 0.01 \)) (Fig. 3).

During the first 2 days after human OT xenografting, there were no evident signs of neovascularization in either the B-site or K-site grafts, and all of the grafts were negative for anti-mouse CD31 staining (Fig. 4i). Species-specific anti-mouse CD31-positive cells were noticed for the first time in B-grafts on Day 3 and in K-grafts on Day 4 (indicating the start of angiogenesis in the human OTs, derived from mouse origin). On Day 5, the number of OT grafts that were positive for anti-mouse CD31 IHC was significantly higher in the B-site grafts compared with the K-site (40/40 versus 29/40, respectively, \( P = 0.0004 \)) (Fig. 4j and p). After 2 days of grafting, all the grafted OT fragments (B and K) showed a positive staining for anti-human CD31 antibodies (Fig. 4a and b), even though from Day 7 forward, a non-significant decrease was seen in the density of existing mature blood vessels which were stained with \( \alpha \)-SMA (4 ± 0.28 versus 3.6 ± 0.32, respectively) (Fig. 4c).

H&E staining showed that B-site grafts were completely integrated within the surrounding muscle tissue (Fig. 5b–d), whereas the K-site grafts were only partially adhered to the K-site 8 days after grafting (Fig. 5a). Ultrastructural evaluation of the connection points of grafted tissues with the surrounding tissue using EM confirmed our findings (Fig. 5d and e).

Experiment III: the effect of gonadotrophin treatment on graft quality

Angiogenesis was more prominent in the B-site grafts in comparison with the K-site. Administration of FSH led to a significantly higher number of micro-vessels in the grafted tissues in B-grafts during the first 8 days after grafting (15.2 ± 0.89 versus 11.0 ± 0.73, \( P < 0.0001 \)) (Table I). However, this difference was not observed in K-site grafts.

Histomorphological evaluation showed that OTs survived grafting significantly better at FSH-injected B-sites compared with FSH-non-injected K-sites in both short-term (8 days; \( P = 0.01 \)) and long-term xenotransplantation groups (5 months; \( P = 0.047 \)) (Table I). Although a relatively higher number of OTs survived 8 days of grafting at the B-site versus the K-site in both the FSH-injected (30/32 versus 25/32; B and K, respectively) and -non-injected groups (26/32 versus 21/32 respectively), this difference was not statistically significant (Table I). H&E staining and EM demonstrated histologically better tissue quality in B-site grafts after FSH administration (Fig. 5f and g).

Experiment IV: MRI-assisted xenotransplantation

A total of 32 follicles were observed in B-grafts and 21 in K-grafts, with 18 follicles bigger than 4 mm in B-grafts and 2 follicles bigger than 4 mm in K-grafts using our optimized method assisted by MRI (Table II). A significantly higher number of MII oocytes were obtained from MRI-assisted B-site grafts compared with the K-site and control (Table II) (Fig. 6d and e). The number of B-site grafts with more than one antral follicles was also significantly higher (Tables II and III). Additionally, more grafts with multiple follicles were obtained after B-site grafting (9/20 versus 1/20, B versus K site, respectively, \( P = 0.0084 \)) (Table III) (Fig. 6b). Three B-grafts developed two follicles with MII oocytes. Significantly larger antral follicles were obtained from the B-site compared with the K-site in both MRI-assisted and the non-MRI group, but the difference between MRI-assisted B- and
K-site compared with a relevant non-MRI control group was not significant (Fig. 7).

As a result of the subtle pressure from the B-site, in some of the B-site grafts, antral follicles were formed in an oval shape. The transverse axis of such antral follicles was used for our statistical analysis. For example, the biggest graft obtained from MRI assisted-B-site in our experiment contained two antral follicles. The first follicle had a longitudinal axis of 17 mm and a transverse axis of 15 mm. Therefore, 15 mm was used further in our statistical analysis. Other follicles obtained from the same graft had a longitudinal axis of 8 mm and a transverse axis of 6 mm (Table III) (Fig. 6c).

Vascularization was more prominent in B-grafts (number of blood vessels: 11.5 ± 0.64 versus 4.64 ± 0.59, B- versus K-site, respectively, P < 0.001). There was a significant correlation between the number of blood vessels and the size of follicles in the grafts (Spearman’s rank correlation coefficient 0.73; P < 0.001; Fig. 8). Higher numbers of α-SMA-positive vessels were seen in B-site versus K-site grafts (3.96 ± 0.33 versus 1.52 ± 0.25, P < 0.001, respectively) (Fig. 4).

Figure 4 IHC evaluation of angiogenesis in human OT xenografts. (a–d) Anti-human CD31 staining. (a) Existing human blood vessels in OT around the follicles 1 day after grafting. (b) Human blood vessels 1 day after grafting—do not contain blood cells. (c) Reduced number of human blood vessels 12 weeks after grafting. (d) Negative control: the primary antibody was omitted. (e–h) α-SMA. (e) Mature blood vessels 12 weeks after grafting. (f) Ten days after grafting. (g) Mature and functioning blood vessels, 7 months after grafting. (h) Negative control: the primary antibody was omitted. (i–l) Anti-mouse CD31 staining. (i) No cells showing signs of angiogenesis 1 day after grafting. (j) Mouse blood vessels invading xenografted human OT, 7 days after grafting. (k) Functioning mouse blood vessels in human OT graft, 12 weeks after grafting. (l) Negative control: the primary antibody was omitted. (m) * indicates actively functioning blood vessels positively stained with anti-human CD31; ¥ indicates actively functioning blood vessels negative for anti-human CD31. (n) * indicates actively functioning blood vessels positively stained with anti-mouse CD31; ¥ indicates actively functioning blood vessels negative for anti-mouse CD31. (o) M, mouse blood vessels highly stained with anti-mouse CD31; H, human tissue negative for anti-mouse CD31. This slide is a differential control for species-specific anti-mouse CD31. (p) No evidence of angiogenesis is seen 3 days after grafting. Scale bars are μm.
The presence of anastomoses of human and mouse blood vessels in the grafted OTs was demonstrated by finding active blood vessels positive for either anti-human-CD31 or anti-mouse-CD31 (Fig. 4m–o).

A significantly higher number of PCNA-positive follicles was seen in B- compared with K-sites, showing a higher proliferative status of the growing follicles (7.35 ± 0.56 versus 4.75 ± 0.51, \( P = 0.001 \)) (Fig. 3c).

A significant correlation was observed between the number of PCNA-positive follicles and the vascularization of the grafts (Spearman’s rank coefficient = 0.485, \( P < 0.013 \)) (Fig. 3d). Even though a slightly higher number of grafts containing AC-3-positive follicles were seen in the K-site, this finding was not statistically significantly different from B-grafts (\( P = 0.125 \)).

### Table I The effect of gonadotrophin treatment on the survival of grafts and graft neovascularization after xenografting human OT into B- and K-sites of SCID mice.

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<th>Blood vessel count</th>
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<td>8 Days</td>
<td>FSH+ (n = 8)</td>
<td>15.2 ± 0.89&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;,$&lt;/sup&gt;</td>
<td>30/32&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>FSH− (n = 8)</td>
<td>11.0 ± 0.73&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;,$&lt;/sup&gt;</td>
<td>26/32</td>
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<td>5 Months</td>
<td>FSH+ (n = 5)</td>
<td>13.45 ± 0.71&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20/20&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>FSH− (n = 5)</td>
<td>12.26 ± 0.57&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>8 Days</td>
<td>FSH+ (n = 8)</td>
<td>8.1 ± 0.64&lt;sup&gt;*&lt;/sup&gt;</td>
<td>25/32</td>
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<td>FSH− (n = 8)</td>
<td>6.60 ± 0.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21/32&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>5 Months</td>
<td>FSH+ (n = 5)</td>
<td>8 ± 0.47&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>FSH− (n = 5)</td>
<td>6.93 ± 0.44&lt;sup&gt;d&lt;/sup&gt;</td>
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Values are mean ± SEM (n = surviving grafts).
<sup>a,b,c,d</sup>Values with a common letter are significantly different (\( P < 0.05 \), Mann–Whitney U test).
<sup>*</sup>Values with a common letter are significantly different (\( P < 0.05 \), Fisher’s exact test).
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### Competence of oocytes from B-site allografts

One mature oocyte (MII) (Fig. 6g) and one immature oocyte were obtained at the MI stage (Fig. 6f). The immature MI oocyte was subjected to IVM and developed to the MII stage the following day.

### Discussion

The main goal of OT cryopreservation and transplantation is to preserve fertility in young female cancer patients undergoing cytotoxic treatments. The method must allow the grafting of the cryopreserved OT fragments back into the patient years after treatment with a
minimal loss of primordial follicles and with the capability of controlled follicular stimulation and development in the grafted OTs. The cryopreservation step has been well optimized during the last decade using slow freezing (Hovatta et al., 1996). Vitrification was recently introduced as a new technique for OT banking (Isachenko et al., 2008). It is still an ongoing debate as to which transplantation site will result in the best follicular growth and oocyte maturation. Advantages and disadvantages of human OT autotransplantation into different body locations such as the pelvic cavity (orthotopic site) or a heterotopic site, such as the forearm or the abdominal wall (Oktay et al., 2001, 2003; Radford et al., 2001; Donnez et al., 2004; Kim et al., 2004; Meirow et al., 2005; Demeestere et al., 2006), have been discussed. There are limited data available on the quality of oocytes obtained after xenografting. Our previous report demonstrated the feasibility of using OT from female-to-male transsexual persons as research material for ovarian cryopreservation, grafting and tissue culture studies (Van den Broecke et al., 2001). In a preliminary attempt, cryopreserved human OTs from female-to-male transsexuals were used for a series of experiments to optimize handling and thawing methods. Subsequently, human OT fragments were xenotransplanted into mouse B-sites and were compared with the K-site as a conventional grafting site. Early events following grafting were studied because of their crucial impact on the success of transplantation. Ischemic reperfusion injury, resulting from the formation of oxygen-derived free radicals and lipid peroxidation, is the main cause of transplantation failure (Jassem and Heaton, 2004). Any factor minimizing oxidative stress, and consequently ischemia, improves the survival of the primordial follicles in OT grafts (Liu et al., 2002). Extensive follicular damage can occur as a consequence of detachment of the graft from the bloodstream; therefore, rapid neovascularization and reconstructing vascular support are the main goals in xenotransplantation (Aubard et al., 1999; Baird et al., 1999; Oktay et al., 2000; Liu et al., 2002). It is obvious that the transplantation site has a vital impact on primordial follicle survival and final development to the antral stage by providing sufficient blood supply to the tissue (Soleimani et al., 2008). Allografting mouse hemi-ovaries into B-sites significantly improved primordial follicle survival after grafting as a result of rapid and considerable

### Table II Characteristics of individual follicles following xenotransplantation of human OT fragments into B- and K-sites.

<table>
<thead>
<tr>
<th>B</th>
<th>Mouse no.</th>
<th>Number of follicles</th>
<th>Follicle size (mm)</th>
<th>Oocyte stage</th>
<th>Mouse no.</th>
<th>Number of follicles</th>
<th>Follicle size (mm)</th>
<th>Oocyte stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>1</td>
<td>3</td>
<td>GV</td>
<td>21</td>
<td>1</td>
<td>3</td>
<td>MII</td>
</tr>
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<td>4.5</td>
<td>MII</td>
<td>22</td>
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<td>1</td>
<td>GV</td>
</tr>
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<td>1</td>
<td>1</td>
<td>GV</td>
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<td>GV</td>
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<td>MII</td>
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<td>6</td>
<td>MII</td>
</tr>
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<td>MII</td>
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<td>1.5</td>
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<td>GV</td>
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<td>2</td>
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<td>MII</td>
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<td>2</td>
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<td>1</td>
<td>1.5</td>
<td>No oocyte</td>
<td>34</td>
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<td>1</td>
<td>GV</td>
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<td>1.5</td>
<td>GV</td>
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<td>GV</td>
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<td>1</td>
<td>4</td>
<td>GV</td>
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<td>1.5</td>
<td>GV</td>
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<td>GV, MII</td>
<td>39</td>
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<td>40</td>
<td>1</td>
<td>1.5</td>
<td>GV</td>
<td></td>
</tr>
<tr>
<td>Mean number of follicles/graft</td>
<td>1.60 ± 0.18*</td>
<td>Mean number of follicles/graft</td>
<td>1.05 ± 0.05*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>K</th>
<th>Mouse no.</th>
<th>Number of follicles</th>
<th>Follicle size (mm)</th>
<th>Oocyte stage</th>
<th>Mouse no.</th>
<th>Number of follicles</th>
<th>Follicle size (mm)</th>
<th>Oocyte stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>3</td>
<td>11</td>
<td>GV</td>
<td>7</td>
<td>4</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*P = 0.028 (Mann–Whitney U test).

MII/MII, metaphase I/II oocyte; GV, germinal vesicle.
neovascularization in the grafted tissue (Soleimani et al., 2008). The B-site, as introduced in the present study, showed excellent capabilities in supporting human OT grafts, starting from early grafting stages. Prompt neovascularization led to the rapid integration of the grafts in the muscle tissue during the first few days after grafting. OT grafts were totally embedded in the muscle tissue, and mechanical separation of both was difficult 1 week after grafting. Good neovascularization with a higher number of blood vessels in B-site led to the formation of a bigger antrum. Ease of separation of the grafted OTs at the K-site 1 week after grafting may be considered one of the limitations of the K-site for xenotransplantation studies. Lack of contact of the xenografted OT fragments with the kidney may result from the lack of sufficient pressure from the capsule. Some of the K-site grafts were surrounded by adipose tissue after long-term grafting.

Others have attempted to reduce ischemia-related damage of transplantation by the administration of GnRH (Imthurn et al., 2000) or by using antioxidants (Nugent et al., 1998). Consequently, we also studied the effect of gonadotrophin administration on the graft quality. Administration of FSH immediately after grafting resulted in better neovascularization at the B-site. The improvement in graft quality in FSH-treated animals in the long term and the significant difference in graft survival in B-site compared with the K-site in non-FSH-treated controls encouraged us to administer FSH during the whole period of grafting.

Remarkably, detectable mouse blood vessels started to invade the grafted human OT into the B-site from Day 3 onward. This step was visualized by means of species-specific anti-mouse and anti-human CD31 IHC staining in histological sections from the grafts.

Table III Comparison of the results obtained in Experiment IV in B- and K-site xenotransplantation versus non-MRI visualized group.

<table>
<thead>
<tr>
<th></th>
<th>Non-MRI</th>
<th>MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>K</td>
</tr>
<tr>
<td>Number of follicles &gt; 4mm/total (%)</td>
<td>8/22 (36)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/22 (0)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Number of grafts with minimum one MII follicle/total (%)</td>
<td>4/22 (18)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2/22 (9)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Number of grafts with multiple follicles/total (%)</td>
<td>3/22 (14)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1/22 (5)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean follicle size (mm)</td>
<td>4.11 ± 0.58&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.27 ± 0.18&lt;sup&gt;b,h&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maximum follicle size (mm)</td>
<td>12 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.5</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d,e</sup>Values with the same letter in the same row are significantly different (P < 0.05, Fisher’s exact test).

<sup>g,h,i</sup>Values with the same letter in the same row are significantly different (P < 0.005, Mann–Whitney U test).

Figure 6 Macroscopic analysis of B-site grafts. (a) Antral follicle containing a germinal vesicle oocyte. (b) Graft with multiple antral follicles containing oocytes. (c) OT recovered after 7 months. (d) Antral follicle containing an MII oocyte. (e) Higher magnification of the MII oocyte from (d) with a visible polar body. (f) MII oocyte obtained from B-site grafting. This oocyte became MII after IVM. (g) MII oocyte obtained from B-site grafting.
In general, vascularization (both from human and murine origin) was delayed and was less extended in K-site compared with B-site grafts. Interestingly, in the long term, invading murine vessels made anastomoses with existing human blood vessels in the transplanted OT and supplied blood from murine origin to the grafts. This supportive nature of the B-site resulted in higher numbers of growing follicles positive for PCNA IHC and lower number of follicles positive for AC-3 IHC, indicating prevention of ischemia. There was also a significant positive correlation between the number of blood vessels in the grafted tissue and the size of the antral follicles obtained at the end of

**Figure 7** Visualization of growing follicles after grafting, using MRI. (a) Box designed for restraining the animals for MRI. (b) Fast MRI, scanning of the animals to position them correctly before final imaging. (c) Antral follicle visualized by MRI, grafted into B-site. (d) Macroscopic image of the same follicle as (c) after recovery. (e) Growing follicle in B-site OT graft, visualized by ultrasonography (scale bar 4.6 mm). Please notice that owing to oval shape of the follicle, the transverse axis of the follicle (scale bar 4.6 mm) was recorded as the actual size (longitudinal axis in this follicle is 6.5 mm).

**Figure 8** Human follicle size and vascularization of the follicles obtained after B-site (open squares) or K-site (solid squares) grafting in mice. Data are mean ± SD. The correlation of the size of the follicles with the number of blood vessels (Spearman’s rank correlation coefficient = 0.73) is highly significant (P < 0.001).
the experiment. Quantitatively improved and faster neo-
vascularization at B-sites appears to be key for achieving good
quality follicles after grafting. Israely et al. (2006) demonstrated
improvement of follicular survival and better support of OT grafts
after transplanting them into angiogenic granulation tissue in the
area of a healing wound.

The present study was a successful effort to improve the human OT
xenografting model in SCID mice. The novel technique of stereomi-
crosscopical pre-evaluation of follicular content of the grafts before
transplantation proved that not all the fragments were suitable for
xenotransplantation. In our experiments, and based on our selection
criteria (at least five visible follicles in each small OT fragment), only
\(~60\%\) of OT fragments were suitable for grafting. This simple eval-
uation led to a significant improvement of the results and can be
highly beneficial for both clinical and research trials. It has been
reported that, in the natural human menstrual cycle, once selection
of the leading follicle has occurred, the developmental potential of
the oocytes is impaired (Cobo et al., 1999). In the comparative
study of B and K-sites, we xenotransplanted one piece of OT into
either the B or K-site in SCID animals to prevent the effect of the
dominant follicle, in order to minimize the difference between experi-
mental groups. A similar situation can be extrapolated to the grafting
of more than one OT fragment to each SCID mouse to obtain mature
oocytes in the long term. On the other hand, as the actual size of
human antral follicles is relatively large compared with that in a
mouse, grafting more than one OT fragment can have an impact on
animal welfare.

Graft size is also a very important issue during transplantation.
Bigger and thicker tissues need more time to be invaded by murine
blood vessels and this can result in follicular loss due to ischemic–
reperfusion injury during the generation of new blood vessels in the
grafts. Ischemic–reperfusion injury after xenotransplantation can be
prevented by grafting small and thin OT fragments. Our preliminary
study showed that a size of \(1 \times 1 \text{ mm}^2\) and \(<0.5 \text{ mm thickness}\) is
optimal (data not shown).

Even though Experiment I in our study showed no difference in the
number of antral and pre-antral follicles between B- and K-sites, we
could obtain larger follicles after B-site grafting. This indicates the
potential of the B-site for developing mature oocytes. Morphological
and physiological changes in antral follicles are strongly dependent
on the initiation of the meiotic maturation process in the enclosed
oocytes. Simple morphological changes provide a quantitative and
selective indicator of gonadotrophic action within the ovary (Schuetz
and Swartz, 1979). Obtaining a follicle of \(~18 \text{ mm}\) in size is one of
the main clinical goals during oocyte maturation, and smaller antral fol-
licles normally respond poorly to hormonal stimulation (Grondahl,
2008). FSH is one of the main survival factors for early antral follicles.
During this stage, most follicles go through atresia under physiological
circumstances (Chun et al., 1996). Our findings support the report
that FSH can stimulate granulosa cell mitosis and follicular maturation
and inhibits granulosa cell apoptosis after the initial stages (Gougeon,
1996).

In the present study, the antral follicle sizes obtained from
FSH-stimulated xenografts into the B-site of female SCID mice are
relatively larger than those previously reported following grafting of
fresh human OT either into K-sites (2.5–5 mm) (Oktay et al., 1998)
or s.c. (6 mm) (Weissman et al., 1999) or cryopreserved human
OT grafted in K-sites (1–6 mm) (Gook et al., 2003) in female
recipients. Although Hernandez-Fonseca et al. (2003) reported large
antral follicle development after xenotransplantation of human OT
into the K-site (7 mm) or s.c. (15 mm) (Hernandez-Fonseca
et al., 2004) in castrated male mice, transplanting human OT into
males cannot correspond to the conditions of the female body.
Massive follicle loss after xenografting into s.c. sites has been reported
(Weissman et al., 1999). Others, including Hernandez-Fonseca et al.
(2004), showed that K-site grafting leads to higher numbers of
growing follicles compared with s.c. (Abir et al., 2003). The K-site
has been shown to be superior to the s.c. site in supporting fresh or
frozen human OT grafts (Abir et al., 2003). Our previous study of allo-
grafting mouse hemi-ovaries into different body locations (B-, K-
and s.c. sites) showed the advantage of the B-site because higher
numbers of grafts survived the transplantation, whereas we could
only recover two grafts out of six in s.c. sites (Soleimani et al., 2008).

Even though the evaluation of antrum formation after grafting was
also possible using ultrasonography, it was time consuming and it
was practically difficult owing to the high number of animals to
examine each time. Therefore, we used MRI to monitor and evaluate
the size of antral follicles in the grafted tissues. Our chamber, specially
designed for restraining the mice during MRI, had the advantage of
saving time during the visualization.

In conclusion, a high number of mature MII oocytes were obtained
by performing stereomicroscopic evaluation of baseline follicular
content and using MRI to evaluate OT grafts to assess follicular
growth by measuring antral follicle size before final HCG stimulation.
Obtaining a higher number of grafts with more than one antral follicle
was one of the advantages of B-site grafting. Further studies are
required to characterize MII oocyte quality obtained after xenotrans-
plantation. Additionally, ease of manipulation is an extra advantage of
the B-site. To our knowledge, this is the first report of an MII and
IVM–MII oocyte being obtained from a human antral follicle after
xenografting to the B-site. (MII oocytes recovered during complemen-
tary studies were not fertilized owing to ethical restrictions.) The
present study demonstrates the advantages of muscle tissue for the
development of large antral follicles containing mature MII oocytes
in a mouse xenografting model. We here report the largest size of fol-
licles containing mature oocytes obtained from human to mouse OT
xenotransplantation to any site, and in particular to the B-site, to date.

The murine B-tissue model is promising for further exploration as a
model for human OT autotransplantation.

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and Health Sciences, Ghent University Hospital for their kind assist-
ance in preparation of histological sections, in particular Mrs Dorothea
Van Limbergen.

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