

The best source of isolated stromal cells for the artificial ovary: medulla or cortex, cryopreserved or fresh?

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STUDY QUESTION: What is the best source of ovarian cells for the artificial ovary: medulla or cortex, cryopreserved or fresh?

SUMMARY ANSWER: Ovarian cells from fresh medullary tissue, which can be isolated in larger numbers, show higher viability and are able to improve graft vascularization.

WHAT IS KNOWN ALREADY: In a previous study, addition of endothelial cells along with ovarian cells was found to be crucial for formation of a well-vascularized ovary-like structure. This study is the first to evaluate both the effect of cryopreservation and the source of ovarian tissue on isolated ovarian cells.

STUDY DESIGN, SIZE, DURATION: Prospective experimental study in an academic research unit using ovarian tissue from seven patients undergoing surgery for benign gynecologic disease.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Ovarian tissue was retrieved from seven patients, with one half processed as fresh (fresh group) and the other half frozen and thawed before processing (frozen group). In each group, ovarian cells from the cortex and medulla were isolated separately, and their viability was tested using a calcein AM/ethidium homodimer viability assay. Fifty thousand cells were then encapsulated in fibrin and grafted to peritoneal pockets in nude mice (14 in all). Grafts recovered after 7 days were analyzed by immunohistochemistry for the presence of ovarian cells (vimentin), proliferation (Ki67) and graft vascularization (double CD34). Cell apoptosis was analyzed by TUNEL assay.

MAIN RESULTS AND THE ROLE OF CHANCE: Cryopreservation decreased ovarian cell yield (-2804 cells/mg, $P = 0.015$) and viability (-9.72% , $P = 0.052$) before grafting and had a considerable (5-fold, $P = 0.2$) but non-significant negative impact on ovarian cell presence in grafts. The medulla yielded many more cells ($+3841$ cells/mg, $P < 0.001$) with higher viability ($+18.23\%$, $P < 0.001$) than did the cortex. Moreover, grafts with cells from the medulla exhibited a statistically significant 6.44- and 2.47-fold increase in human and total vascular surface area, respectively. P -values were adjusted for multiple testing using the Benjamini–Hochberg method to achieve a 10% false discovery rate and adjusted P -values < 0.1 were therefore considered significant.

LIMITATIONS, REASONS FOR CAUTION: Pilot study involving a limited number of experiments.

WIDER IMPLICATIONS OF THE FINDINGS: Knowing that fresh medullary tissue is the best source of stromal cells is important for construction of the artificial ovary, as isolated follicles require structural support and a rich vascular network for their survival and development.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by grants from the Fonds National de la Recherche Scientifique de Belgique (5/4/150/5 and 7.4518.12F), Fonds Spéciaux de Recherche, Fondation Saint Luc and Foundation Against Cancer, and donations from Mr Pietro Ferrero, Baron Frère and Viscount Philippe de Spoelberch. None of the authors have any conflicting interests to declare.

Key words: artificial ovary / isolated ovarian cells / ovarian tissue / cryopreservation / cancer patients

Introduction

Growing numbers of young girls and women are now surviving cancer, with an increase in 5-year relative survival rates from around 60% in 1975 to 82–86% in 2009 (Edwards et al., 2013). Unfortunately, one of the long-term consequences of these ever more effective treatments is destruction of the ovarian follicular reserve, with possible premature menopause and subsequent infertility (Meirow and Nugent, 2001; Wallace et al., 2005; Donnez et al., 2006; Anderson and Wallace, 2013). This has become an important quality-of-life issue for the increasing population of cancer survivors, making fertility preservation a priority concern for these patients once cured of their disease.

Patient age, cancer type and planned treatment, time available for implementation of fertility preservation methods, patient relationship status and the likelihood of ovarian metastasis are all elements to be considered in the fertility preservation decision and approach (Donnez and Dolmans, 2013; De Vos et al., 2014). Current clinical options for female fertility preservation before gonadotoxic treatment (apart from ovariopexy for patients undergoing pelvic irradiation) include embryo and mature oocyte cryopreservation following ovarian stimulation (Kim et al., 2012; Donnez and Dolmans, 2013; De Vos et al., 2014; de Lambert et al., 2015). These strategies are not, however, applicable to prepubertal patients and those requiring immediate chemotherapy, which has led to the development of an alternative technique: ovarian tissue cryopreservation before cancer treatment. Reimplantation of frozen-thawed ovarian tissue after gonadotoxic treatment has proved its worth, with more than 40 live births worldwide to date (Donnez et al., 2013; Donnez and Dolmans, 2014; Donnez et al., 2015).

An important concern when contemplating transplantation of cryopreserved ovarian tissue is the risk of ovarian involvement in certain types of cancer, and hence possible reintroduction of malignant cells via the graft (Meirow et al., 2008; Abir et al., 2010, 2014; Amiot et al., 2013; Bastings et al., 2013; Dolmans et al., 2013; Luyckx et al., 2013b; Rosendahl et al., 2013). Indeed, experimental studies on patients with acute leukemia have demonstrated by sensitive polymerase chain reaction methods that cryopreserved ovarian tissue may harbor leukemic cells in >50% of cases (Meirow et al., 2008; Dolmans et al., 2010; Rosendahl et al., 2010) and therefore transmit the disease, at least in a xenografting model (Dolmans et al., 2010). For patients with such a pathology, transplantation of frozen-thawed ovarian tissue is not currently recommended.

For these patients at risk of minimal disseminated disease (MDD), follicle culture with *in vitro* maturation may be a solution to restore fertility without the danger of reseeding cancer (Telfer et al., 2008; Telfer and McLaughlin, 2011; Telfer and Zelinski, 2013). However, despite advances in this field, viable embryos and live off springs have only been obtained in mice with this technique (Spears et al., 1994; Eppig and O'Brien, 1996; O'Brien et al., 2003), and major hurdles remain before improved culture systems in human are able to yield competent human oocytes (Telfer and Zelinski, 2013; Shea et al., 2014). Another technique, yet experimental for young patients undergoing ovarian cryopreservation could be supplementary immature oocyte aspiration from the cortex, followed by *in vitro* maturation and cryopreservation of mature oocytes (Revel et al., 2009; Fasano et al., 2011; Prasath et al., 2014).

Grafting of pre-antral follicles enzymatically isolated from frozen-thawed ovarian tissue and embedded in a 3D matrix along with other ovarian cells, what we call an 'artificial ovary', is a way of restoring fertility using follicles present in a patient's cryopreserved ovarian tissue

(Dolmans et al., 2007; Dolmans et al., 2008; Vanacker et al., 2012; Luyckx et al., 2013a, 2014). This artificial ovary provides a more natural environment for follicle survival and development *in vivo*, and the first studies are encouraging (Dolmans et al., 2008; Luyckx et al., 2014).

Ovarian stromal cells have been shown to secrete various factors that positively regulate primordial-to-primary follicle transition (Nilsson and Skinner, 2003; Lee et al., 2004; Knight and Glister, 2006) and are recruited by growing follicles to differentiate into steroid-secreting theca cells required for follicle development and ovulation (Magoffin and Magarelli, 1995; Magoffin, 2005). Moreover, addition of endothelial cells was found to be crucial for formation of a well-vascularized ovary-like structure (Dath et al., 2011). Integration of ovarian cells into the artificial ovary could thus serve to better simulate the ovarian microenvironment and potentially improve follicle growth and survival.

Ovarian cells (stromal and endothelial) for the artificial ovary could be isolated, like follicles, from ovarian cortex cryopreserved before chemotherapy. In this case, stromal cells would have to undergo malignant cell purging before they could be safely used. An alternative is to take a fresh ovarian biopsy (cortex and/or medulla) just before transplantation of the artificial ovary, once the patient is disease-free. These cells isolated from fresh ovarian tissue would then be combined with follicles obtained from cryopreserved cortex and would not require malignant cell purging.

The aim of this study was to determine the best source of isolated ovarian cells for the artificial ovary: cortex or medulla, cryopreserved or fresh. To this end, the impact of freezing and thawing procedures, as well as the cortical or medullary origin of ovarian tissue, was first evaluated by comparing stromal cell yield and viability after isolation. Then, by grafting these stromal cells to mice in a fibrin matrix for 1 week, the influence of these factors on *in vivo* ovarian cell presence, proliferation and graft revascularization could be analyzed.

Materials and Methods

The experimental design is shown in Fig. 1. A series of seven experiments were carried out using human ovarian tissue from seven premenopausal patients (34–49 years of age) undergoing laparoscopic surgery for benign gynecologic disease, after obtaining informed consent. Patient and biopsy characteristics are provided in Supplementary Table S1. Use of human tissue for this study was approved by the Institutional Review Board of the Université Catholique de Louvain (IRB, 2012, 125).

Processing of ovarian tissue

Each biopsy was divided into two halves destined for two different groups: a 'fresh' group (cortex and medulla) processed on the same day, and a 'frozen-thawed' group (cortex and medulla) cryopreserved immediately and thawed a few days later for processing. The slow-freezing and thawing protocols used were previously described by our team (Amorim et al., 2009; Dolmans et al., 2010). Briefly, the ovarian fragments were transferred to cryovials containing freezing medium with dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) as a cryoprotectant. The cryotubes were placed in a programmable freezer (Freeze Control CL-8800i; CryoLogic, Victoria, Australia) and cooled using a slow-freezing program for ovarian tissue, before being stored at -196°C in liquid nitrogen.

For each group, fresh and frozen, the cortex was separated from the medulla using surgical scissors, and both were weighed and processed independently. Tissue was digested using Liberase DH (Roche Diagnostics, Mannheim, Germany), as previously described (Vanacker et al., 2011). The resulting cell suspension was successively filtered through sterilized 80 and

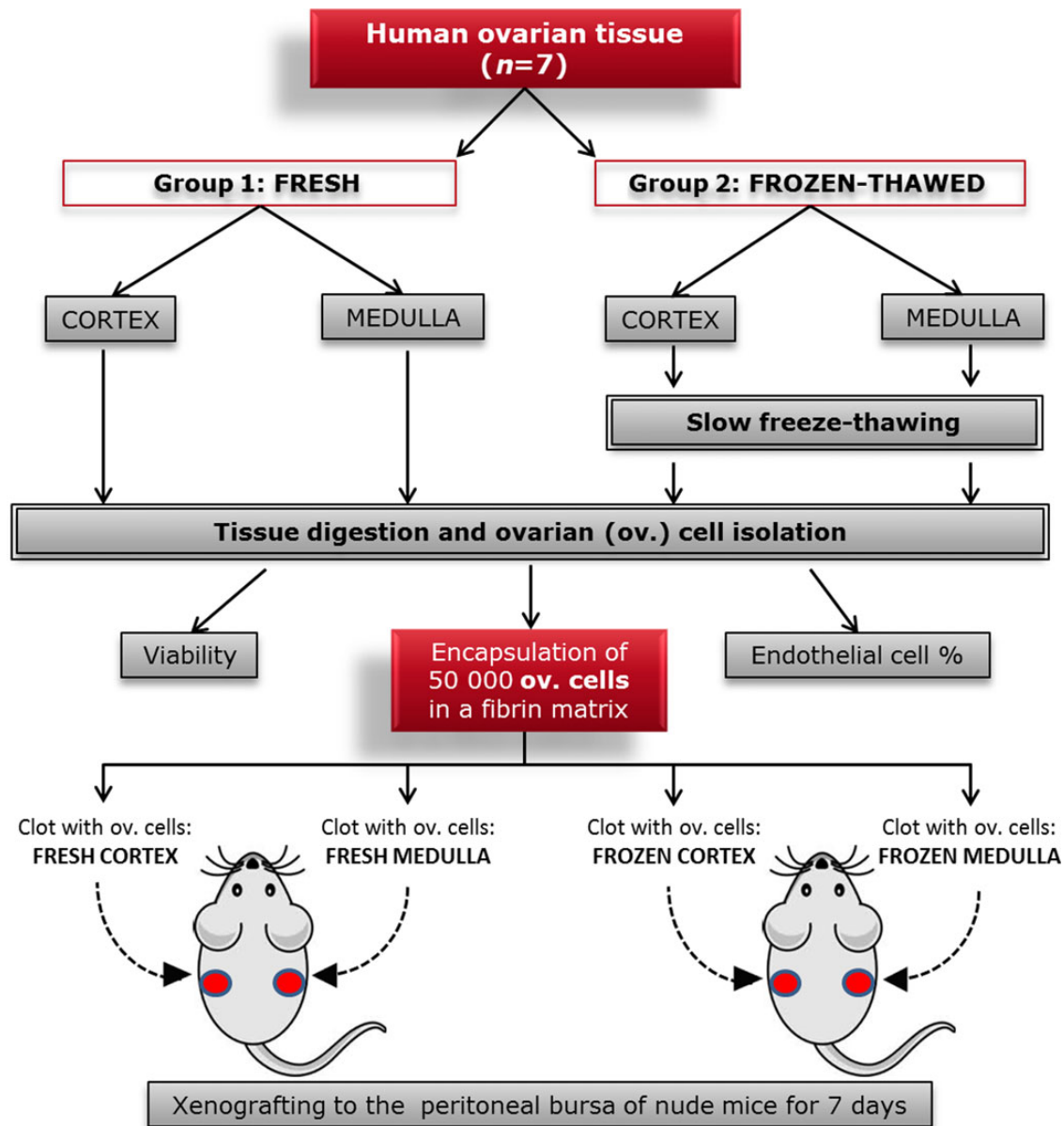


Figure 1 Experimental design.

11 μm nylon net filters (Millipore, Brussels, Belgium). The solution obtained was centrifuged (240 g, 5 min) and the pellet resuspended in 500 μl of Dulbecco's phosphate-buffered saline (PBS, Gibco, Life technologies) supplemented with 10% (v/v) fetal bovine serum (Gibco). Cells were counted using a Bürker chamber and concentrated by centrifugation in order to obtain 50 000 cells/5 μl , after first retrieving a few microliters of suspension for the viability test ('Ovarian cell viability after isolation').

Fibrin clot formation

Reconstitution and dilution of the two components of the fibrin sealant (Tissucol, Baxter, Lessines, Belgium) and fibrin clot formation have been previously described (Luyckx *et al.*, 2013a). Briefly, a droplet of 12.5 μl fibrinogen (12.5 mg/ml) was deposited on a glass petri dish and 50 000 human ovarian cells (5 μl) were added. The fibrinogen containing the cells

was then mixed with 12.5 μl thrombin (1 UI/ml) on a plastic petri dish and incubated at 37°C for 45 min for fibrin polymerization. The fibrin clot was then gently detached and grafted to mice.

Grafting fibrin clots to nude mice

Guidelines for animal welfare were approved by the Committee on Animal Research of the Université Catholique de Louvain. Fourteen 6- to 10-week-old nude female mice (Swiss nu/nu, Charles River Laboratories, France) were used for this study. Their housing conditions, anesthesia and analgesia were previously described (Vanacker *et al.*, 2012; Luyckx *et al.*, 2014). A ventral midline incision was made and a peritoneal pocket was created on the inner side of the peritoneum using non-absorbable 6-0 Prolene suture. The fibrin clot was gently pushed into the peritoneal pocket, which was closed with a stitch. Each mouse was grafted with a fibrin clot containing

50 000 cells isolated from ovarian cortex on the right and medulla on the left. Atipamezole (1 mg/kg; Pfizer, Brussels, Belgium) was administered after surgery in order to reverse anesthesia.

After 1 week, the mice were euthanized by cervical dislocation and the recovered grafts fixed in 4% (v/v) formaldehyde.

Analyses performed on isolated ovarian cells

Ovarian cell viability after isolation

A viability test (calcein AM/ethidium homodimer; Molecular Probes, Leyden, the Netherlands) was performed to evaluate isolated cell viability using a previously described protocol (Martinez-Madrid et al., 2004). A few microliters of the ovarian cell suspension (2000–3000 cells) were transferred to 20 μ l PBS containing 2 mmol/l calcein AM and 5 mmol/l ethidium homodimer and incubated for 30 min at 37°C in the dark, before being observed under a fluorescence microscope (Leica, Van Hopplynus Instruments, Germany). Sections were digitized, either for calcein or ethidium homodimer staining, using a Leica DFC320 camera and IM50 program (Leica). Manual counting of all live and dead cells was performed in Image J 1.46r (NIH, USA).

Characterizing the endothelial cell population

Excess ovarian cells were fixed in 4% (v/v) formaldehyde for 1 h, and projected on glass slides (Superfrost Plus, Menzel-Glaser, Germany) in monolayer cell preparations using the Thermo Electron Cytospin 2 centrifuge (Fisher Scientific, Brussels, Belgium). Anti-human CD34 immunostaining was performed in order to evaluate the endothelial cell proportion in ovarian cells for each group after isolation, according to an immunostaining protocol previously described by Dath et al. (2011). The primary antibody used was mouse anti-human CD34 (1/5000, clone QBend/10; Biocare Medical, Duiven, Belgium). Vascular endothelial cells in human ovarian tissue were used as positive controls. For negative controls, the slides were incubated without the primary antibody. They were then scanned using Mirax Scan (Zeiss, Germany) and visualized with Mirax Viewer software. Three squares of area 1.88 mm² were delimited and all positive and negative cells contained in these squares were manually counted.

Analyses performed on grafts recovered on day 7

After fixation, grafts recovered on day 7 were dehydrated, embedded in paraffin and serially sectioned (5 μ m-thick sections). Every fourth slide was stained with hematoxylin–eosin (Merck, Germany) for histological evaluation: localization of the grafted matrix and observation of graft degradation and vascularization. The remaining slides (Superfrost Plus, Menzel-Glaser, Germany) were kept for further investigations.

Analyses described below were performed on two slides (eight sections) representative of each graft at histology, with adjacent slides used for the different evaluations. For immunostaining, the slides were first deparaffinized with Histosafe and rehydrated in 2-propanol. Immunostaining protocols were the same as those described in the ‘Characterizing the endothelial cell population’ section. For negative controls, the slides were incubated without the primary antibody.

Presence of grafted ovarian cells

Species-specific anti-human vimentin immunohistochemistry (IHC) was performed in order to mark grafted ovarian cells and distinguish them from murine cells invading the grafts. Vimentin, a cytoskeletal protein, marks cells of mesenchymal origin and is strongly expressed in ovarian stromal cells (Czer-nobitsky et al., 1985). Mouse anti-human vimentin was used as the primary antibody (1:200 dilution, clone V9, Dako, Heverlee, Belgium) and human ovarian tissue as a positive control. Two slides per graft were scanned using the Mirax Scan system (Zeiss, Germany) and analyzed with Mirax Viewer software.

After graft delimitation, vimentin-positive areas were determined in each graft and the percentage of vimentin-positive surface area was calculated.

Proliferation of grafted cells

The Ki67 protein is a cellular marker of proliferation, absent in resting cells (G0 phase) but present during all active phases of the cell cycle (G1, S, G2 and mitosis). Proliferation of grafted cells in clots was analyzed by IHC using mouse anti-human Ki67 as the primary antibody (1:100 dilution, clone MIB1, ref M7240, Dako) and Envision anti-mouse (1:2 dilution, Dako) as the secondary antibody. Proliferative endometrium was used as a positive control. This tissue was obtained from our university biolibrary. Use of human tissue from the biolibrary was approved by the Institutional Review Board of the Université Catholique de Louvain (IRB, 2008, 150; EudraCT number 2008/001805–40). The slides were scanned using Mirax Scan and analyzed with Mirax Viewer software. Grafts were manually delimited and the number of Ki67-positive cells was counted.

Apoptosis

Apoptosis in grafts was analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), according to a previously described protocol (Martinez-Madrid et al., 2007). The In Situ Cell Death Detection Kit, TMR red (Roche Applied Science), was used to detect strand breaks of DNA occurring during the apoptotic process. Human tonsil tissue was used as a positive control and negative control sections were incubated with label solution without enzyme solution. Finally, the slides were covered with Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA), coverslipped and sealed around the perimeter with nail polish, stored at 4°C, and protected from light. They were then scanned using the Mirax Scan apparatus, which showed an excitation wavelength in the range of 520–560 nm (red fluorescence), and emitted light at a wavelength between 570 and 620 nm. DAPI reached excitation at $\sim$ 360 nm and emitted light at $\sim$ 460 nm when bound to DNA, producing blue fluorescence in all nuclei. TUNEL-positive (red) and total (blue) cells were counted using Image J software.

Graft vascularization

Double anti-human and anti-mouse CD34 IHC was performed in order to concomitantly visualize human and murine vessels in the grafts and analyze graft vascularization. After incubation with the first primary antibody, rat anti-mouse CD34 (dilution 1:100, Hycult Biotechn, Uden, the Netherlands), for 1 h at room temperature, the slides were incubated for 60 min at room temperature with the first secondary antibody, rabbit anti-rat, coupled to biotin (Vector). Diaminobenzidine (Dako) was used as a chromogen after incubation with solution of streptavidin–horseradish peroxidase for 30 min. The slides were incubated again with goat serum to block non-specific binding sites for 30 min, before incubation with the second (anti-human) primary antibody, mouse anti-human CD34 (1:500 dilution, Biocare Medical), overnight at 4°C. Sections were then incubated with the second secondary antibody, goat anti-mouse (1:300 dilution; Jackson ImmunoResearch, Suffolk, UK). Fast Red TR (Sigma) was used as a chromogen and nuclei were counterstained with hematoxylin. Human ovarian tissue grafted to mice for 7 days was used as a positive control. Once again, the slides were scanned using Mirax Scan and analyzed with Mirax Viewer software. Clot surface area was manually delimited and human, murine and chimeric vessels in grafts were manually determined and counted.

Statistical analysis

Statistical analyses were performed using R statistical software (version 3.1.2, R Statistical Computing, Vienna, Austria). The impact of cryopreservation (frozen versus fresh) and the source of ovarian cells (medulla versus cortex) on each outcome were assessed using a mixed-effect linear regression model. Mixed-effect models are particularly suited to analyzing our data because multiple measurements were carried out on each patient.

Indeed, such models take into account the correlation structure between these measurements, in contrast to analysis of variance and standard regression models. In the model used, the 'patient effect' was assumed to follow normal distribution and was therefore defined as a random effect, while cryopreservation and cell origin were taken as fixed effects (Brown and Prescott, 2006). *P*-values obtained were adjusted for multiple testing using the Benjamini–Hochberg method to achieve a 10% false discovery rate (FDR). Benjamini–Hochberg-adjusted *P*-values < 0.1 were therefore considered significant. Residual plots were inspected for each model and log10 transformation was applied to some outcomes in order to stabilize variance. For these outcomes, back-transformation of the model coefficients thus yielded multiplicative effects of cryopreservation (frozen versus fresh) and the source of ovarian cells (medulla versus cortex).

Results

Table 1 shows the results of the effect of cryopreservation (compared with fresh tissue) and the medullary (versus cortical) origin of tissue on the different parameters studied.

Analyses performed on isolated ovarian cells

Ovarian cell yield

The number of ovarian cells isolated per milligram of tissue (cell yield) was calculated for each group. In the fresh cortex group, the average cell yield was 3115.0 cells/mg of tissue (Fig. 2A). Compared with this reference group, cryopreservation of ovarian tissue negatively impacted isolated cell yield (−2804 cells/mg, *P* = 0.015), while the ovarian medulla yielded many more cells (+3841 cells/mg, *P* < 0.001) than the cortex (Table 1 and Fig. 2A). Of the four groups, the frozen cortex group showed the lowest cell yield, some of these samples being particularly poor. Interpatient variations were observed in cell yield but this was not correlated (*r* < 0.25) with patient age.

Isolated cell viability

Ovarian cell viability was assessed before grafting using the calcein AM/ethidium homodimer viability test. All cells were manually counted and the percentage of live cells was calculated. A mean of 3859 (between

Table 1 Results of the impact of cryopreservation (frozen versus fresh tissue) and the source of ovarian cells (medulla versus cortex) on each outcome assessed using a mixed-effect linear regression model.

	Variables analyzed in the original scale: additive effect			
	Effect of cryopreservation (versus fresh tissue)		Effect of medullary origin (versus cortical)	
	Estimate (CI 95%)	BH-adj. <i>P</i> -value	Estimate (CI 95%)	BH-adj. <i>P</i> -value
Analyses on isolated ovarian cells				
Ovarian cell yield (cells/mg tissue)	−2804.5 (−4226.3; −1382.7)	0.015	+3841.3 (2392.6; 5236.1)	<0.001
Ovarian cell viability (%)	−9.72 (−16.01; −3.43)	0.052	+18.23 (11.93; 24.52)	<0.001
Endothelial cells (%)	−6.68 (−13.79; 0.43)	0.20	+10.37 (3.69; 17.05)	0.045
Analyses on grafts on day 7				
Cell proliferation (Ki67+ cells/mm ²)	−25.26 (−90.80; 40.28)	0.65	+4.85 (−59.79; 69.49)	0.88
	Variables analyzed in the logarithmic scale: multiplicative effect			
	Effect of cryopreservation (versus fresh tissue)		Effect of medullary origin (versus cortical)	
	Estimate (CI 95%)	BH-adj. <i>P</i> -value	Estimate (CI 95%)	BH-adj. <i>P</i> -value
Analyses on grafts on day 7				
Grafted cell survival (vimentin + graft surface area, %)	0.20 (0.04; 0.85)	0.20	1.77 (0.42; 7.39)	0.55
Apoptosis (TUNEL+ cells %)	0.98 (0.46; 2.06)	0.98	0.79 (0.38; 1.62)	0.56
Graft vascularization				
Human microvessel density (vessels/mm ²)	0.41 (0.11; 1.45)	0.31	4.40 (1.26; 15.45)	0.075
Murine microvessel density (vessels/mm ²)	1.24 (0.69; 2.26)	0.65	1.19 (0.68; 2.09)	0.56
Chimeric microvessel density (vessels/mm ²)	0.27 (0.06; 1.15)	0.21	4.14 (0.97; 17.54)	0.12
Total microvessel density (vessels/mm ²)	1.01 (0.57; 1.78)	0.98	1.75 (0.99; 3.06)	0.12
Graft surface area occupied by human vessels (%)	0.32 (0.07; 1.40)	0.28	6.44 (1.53; 27.18)	0.075
Graft surface area occupied by murine vessels (%)	1.20 (0.64; 2.25)	0.72	1.38 (0.77; 2.48)	0.39
Graft surface area occupied by chimeric vessels (%)	0.31 (0.09; 1.01)	0.20	3.18 (1.00; 10.11)	0.11
Graft surface area occupied by total vessels (%)	0.85 (0.40; 1.81)	0.77	2.47 (1.17; 5.20)	0.075

BH-adj., Benjamini–Hochberg adjusted.

P-values obtained were adjusted for multiple testing using the Benjamini–Hochberg method to achieve a 10% FDR. Benjamini–Hochberg-adjusted *P*-values < 0.1 were therefore considered significant. Statistically significant differences are shown in bold.

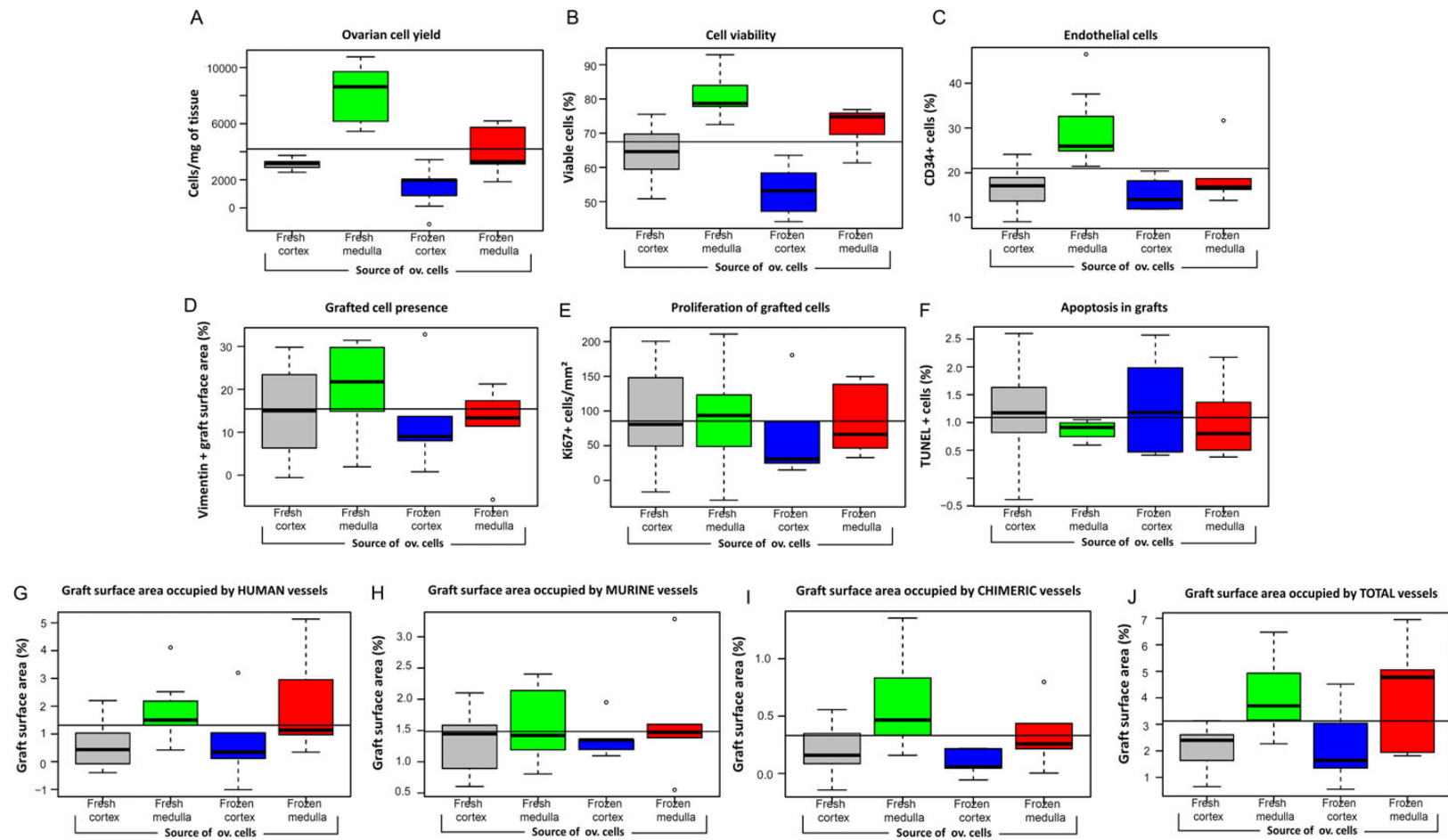


Figure 2 Relative boxplots allow us to visualize the effects of cryopreservation and the stromal cell origin (medulla versus cortex) on each variable. Values were computed for each condition and each patient, relative to the mean patient value across all conditions. The distribution of these relative values is shown in box plots. Values above the horizontal black line (global average computed across all patients and conditions) obtained for a condition means that this condition produces a higher than average value for this patient. (**A–C**) Analyses performed on ovarian cells after isolation. (**D–J**) Analyses performed on the grafts retrieved after 7 days.

1119 and 8821) cells were counted per group and per experiment. Cryopreservation of ovarian tissue was found to have a statistically significant negative impact on cell viability (-9.72% , $P = 0.052$) compared with fresh tissue (Table I). Cells obtained from the ovarian medulla showed increased viability ($+18.23\%$, $P < 0.001$) compared with cells isolated from the cortex. Figure 2B shows that the best results were obtained with fresh medulla and the worst results with frozen cortex.

Endothelial cell population in isolated ovarian cells

Anti-CD34 IHC was performed on slides prepared as described in the 'Characterizing the endothelial cell population' section (cytospins) and a mean of 2875 cells were counted per slide. As expected, a significantly higher number of endothelial cells were found among cells isolated from the ovarian medulla than the ovarian cortex ($+10.37\%$, $P = 0.045$), while the cryopreservation procedure did not have a significant impact on the endothelial cell population (-6.68% , $P = 0.2$) (Fig. 2C).

Analyses performed on grafts recovered on day 7

Histological analysis of grafts showed variable fibrin degradation and generally good graft revascularization (Figs 3A).

Grafted cell survival

Anti-human vimentin IHC (Fig. 3B) enabled us to quantify graft surface area (%) occupied by human cells. This percentage showed wide variation among the four groups and from one experiment to the next

(between 0.2 and 65%). Cryopreserved tissue exhibited a considerable (5-fold) but non-significant relative decrease (fold change: 0.20, $P = 0.2$) compared with fresh tissue. Compared with the cortex, the medulla showed a non-significant increase in graft surface area (%) occupied by human cells (fold change: 1.77, $P = 0.55$) (Table I, Fig. 2D).

Proliferation of grafted ovarian cells

After anti-human Ki67 immunostaining (Fig. 3C), Ki67-positive nuclei were counted and proliferation of grafted human cells was quantified as the number of Ki67-positive cells per mm^2 of graft surface area. Again, the results varied between experiments (from 0.52 to 250.8 positive cells/ mm^2 of tissue). An average of 98.8 Ki67-positive cells/ mm^2 was found in the fresh cortex group. Compared with this reference group, statistical analysis showed a negative but non-significant effect of cryopreservation (-25.26 cells/ mm^2 , $P = 0.65$) on cell proliferation, while isolating cells from the medulla instead of the cortex had an overall positive but non-significant impact on cell proliferation ($+4.85$ cells/ mm^2 , $P = 0.88$) (Table I, Fig. 2E).

Apoptosis

Figure 3D shows TUNEL staining in a graft. For analysis of apoptosis by TUNEL, a minimum of 1000 cells were counted per graft. The apoptotic cell percentage was very low ($<3.3\%$) in all grafts. A very slight and non-significant impact on apoptosis was observed in ovarian cells from cryopreserved tissue (fold change: 0.98, $P = 0.98$) and ovarian cells of medullary origin (fold change: 0.79, $P = 0.56$) (Fig. 2F).

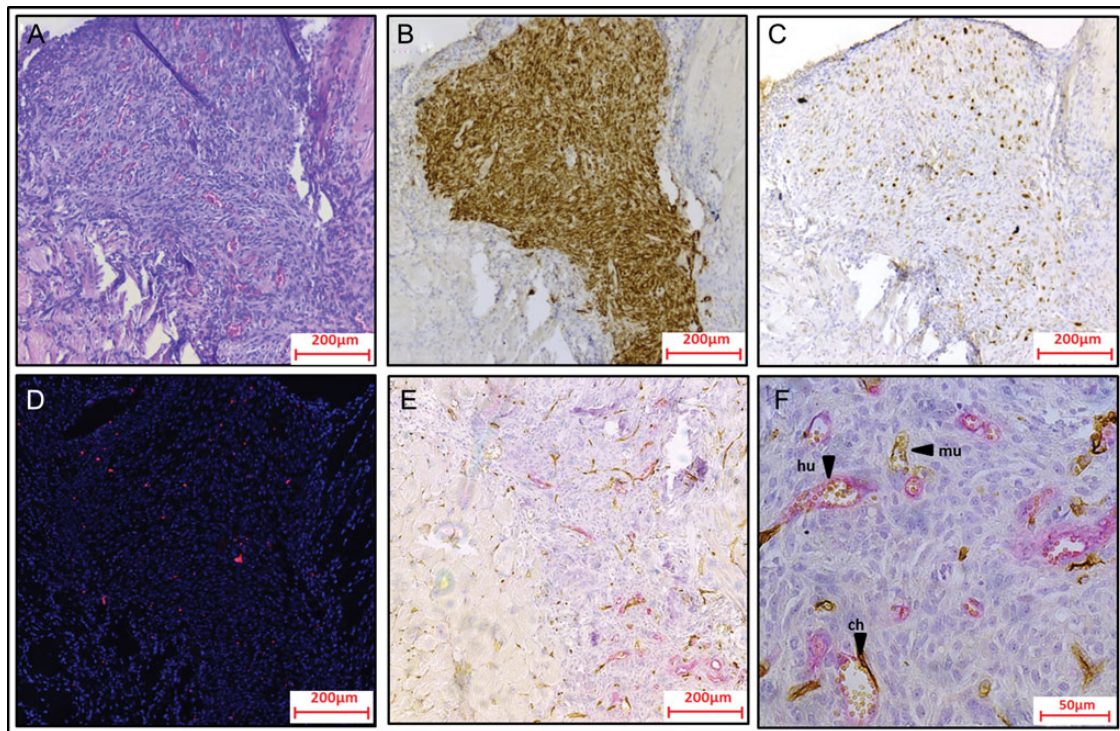


Figure 3 Microscopic and IHC analysis of a clot, with cells isolated from fresh medullary tissue after 7 days of grafting. (A) Histological analysis shows very good clot degradation (all the fibrin has been replaced with cells) and vascularization. (B) Anti-human vimentin IHC was used to localize and quantify human cells in the clot. (C) Proliferating human cells were stained with anti-human Ki67. (D) TUNEL analysis of the clot, with apoptotic cells marked in red. (E and F) Anti-human (red) and anti-murine (brown) double CD34 IHC.

Graft vascularization

Specific human and murine anti-CD34 antibodies were used to investigate the contribution of grafted human endothelial cells and host (murine) cells to graft vascularization. All grafts showed the presence of functional vessels (human and murine) after 7 days, as demonstrated by the presence of intraluminal red blood cells (Fig. 3E and F). Vessel density (vessels/mm² of graft) and the percentage of clot surface area occupied by the different types of vessels were calculated.

Murine vessels contributed widely to graft vascularization on day 7, but this was not influenced by cryopreservation nor by the origin (medulla or cortex) of cells (Fig. 2H).

While cryopreservation did not have any significant impact on human, chimeric or total graft vascularization, use of medullary cells was found to have a positive effect. Compared with grafts with cells of cortical origin, those with cells of medullary origin produced larger clot surface areas occupied by human (fold change: 6.44, $P = 0.075$), chimeric (fold change: 3.18, $P = 0.11$) and total (fold change: 2.47, $P = 0.075$) vessels (Fig. 2G, I and J).

Discussion

For leukemia patients and others at risk of MDD at the time of ovarian tissue cryopreservation, transplantation is not currently recommended. In order to restore fertility in these patients, we aim to develop an artificial ovary that offers a favorable and close-to-natural environment for growth and development of follicles. Addition of ovarian stromal and endothelial cells to the matrix was previously found to be crucial for formation of a well-vascularized ovary-like structure (Dath et al., 2011). This study is the first to evaluate the best source of ovarian cells to be grafted inside the artificial ovary: ovarian cortex or medulla, cryopreserved or fresh. Our findings suggest that cells from fresh medullary tissue can be isolated in larger numbers, show higher viability and serve to improve graft vascularization.

Previous reports have suggested that conventional slow-freezing protocols are detrimental to ovarian cells (Keros et al., 2009; Jin et al., 2010; Sanfilippo et al., 2013). Our study confirms such findings, as we observed that frozen-thawed ovarian tissue yielded smaller numbers of isolated cells with decreased viability. Moreover, if ovarian cells were to be isolated from cryopreserved ovarian tissue, they would first have to undergo a purging step before they could be safely incorporated into the artificial ovary, which would further decrease their viability. Cryopreservation also had a considerable (5-fold) negative, albeit non-significant, influence on the presence of human cells in the grafts, probably due to the limited number of experiments in this pilot study. Inter-individual variations were observed in ovarian cell yield, but this was not correlated with patient age ($r < 0.25$). We found a positive correlation between isolated ovarian cell viability, and presence and proliferation of these cells *in vivo* after grafting. Indeed, a 10% increment in isolated cell viability increased graft surface area occupied by human cells by 7.4% ($P = 0.01$).

Medullary tissue, on the other hand, showed a substantial increase in cell yield and viability compared with cortical tissue, which may be attributed to morphological differences between the cortex (which contains a richly cellular connective tissue stroma) and the medulla (consisting of loose connective tissue and large blood vessels) (Navarini and Reeves, 1972), explaining its easier digestion. Moreover, non-growing primordial

follicles and slow-growing pre-antral follicles do not have a vascular supply of their own, but rather rely on vessels in the surrounding stroma (Stouffer et al., 2001). For the artificial ovary, which uses avascular transplantation of isolated cells to create new functional tissue, rapid neoangiogenesis of the graft is an important prerequisite. The study by Dath et al. (2011) previously showed a correlation between CD34-positive grafted cell percentage and 7-day graft vascularization in plasma clots. Vascular structures of human origin observed in that study as well as the present study probably originate from reorganization of grafted endothelial cells. We showed that the greater number of endothelial cells present among ovarian cells from the medulla is also accompanied by better human and total graft vascularization on day 7. This could decrease the post-grafting ischemia-reperfusion period, thereby increasing cell and follicle survival. This study required use of relatively large biopsies (cortex and medulla) in order to have enough tissue in each of the four groups. Biopsies were therefore taken from older volunteer patients (mean age: 42 years) which is why follicles were not grafted along with ovarian cells.

Our study suggests fresh ovarian medulla as the best source of cells for the artificial ovary. A fresh ovarian biopsy (cortex and/or medulla) could be performed just before transplantation of the artificial ovary, once the patient is disease-free. However, some patients may suffer relapse after being 'cured' of their cancer, which is why a minimum interval of time needs to be respected after cancer remission, and consent obtained from the treating oncologist before transplantation of an artificial ovary, as is currently recommended for ovarian tissue transplantation. Moreover, as opposed to ovarian tissue transplantation where tissue is taken from the patient before treatment, grafting of ovarian cells isolated from a fresh biopsy after cancer cure implies taking and replacing cells that would have remained in the patient's body anyway. Hence, it does not involve 'reintroduction' of untreated and potentially malignant cells.

Chemotherapy, apart from diminishing the primordial follicular pool, is also known to cause stromal cell damage and vascular injury, leading to cortical fibrosis (Marcello et al., 1990; Meirrow et al., 2007). In this study, cryopreserved ovarian tissue was compared with fresh tissue from healthy patients, who had never received chemotherapy. In case of cancer survivors, the fresh ovarian tissue will have undergone several chemotherapy cycles. However, because of the scarcity of such tissue and heterogeneity of chemotherapeutic treatments, it is difficult to conduct a full-scale study on this particular aspect. A preliminary study was carried out (unpublished data) with fresh ovarian tissue retrieved from three patients at the time of ovarian tissue transplantation. All three women had received a number of chemotherapy cycles several years before, during their cancer treatment. Cells isolated from this tissue showed cell yield, viability and *in vitro* proliferation to be similar to fresh controls from women who had not been given any treatment. Chemotherapy does not therefore appear to have a negative impact on isolated ovarian cells.

We found that isolated ovarian cells have the potential to proliferate *in vivo*, degrade the fibrin scaffold and synthesize the extracellular matrix, making them capable of providing isolated follicles with structural support in the artificial ovary. Moreover, some of these cells have the capacity to reorganize into a rich vascular network, which is essential for follicle survival and further development. Unspecialized mesenchymal cells present in the ovarian stroma are known to be recruited by growing follicles (Nilsson and Skinner, 2003; Knight and Glicker, 2006) to differentiate into steroid-secreting theca cells, required for follicle

development and ovulation (Magoffin and Magarelli, 1995; Magoffin, 2005). Our next step will first involve studying the potential of cells isolated from the cortex and medulla to differentiate into theca cells, after which follicles will be xenografted together with ovarian cells. A previous study demonstrated this differentiation capacity with bovine ovarian cells and also reported that stromal cells in the medulla have greater steroidogenic capability than those in the cortex (Orisaka *et al.*, 2006).

Conclusion

Fresh medullary tissue appears to be the best source of ovarian cells for the artificial ovary, thanks to an increased isolated cell yield, better cell viability after isolation and improved graft vascularization. Furthermore, there is no need for malignant cell purging, as the tissue is taken from patients cured of their disease. Integration of these cells into the artificial ovary could thus serve to better simulate the ovarian microenvironment and potentially improve early follicle growth and survival. The next stage will involve studying the effect of these isolated cells on follicle survival and development in the artificial ovary.

Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

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Authors' roles

M.S.: study design, experimental procedures, analyses, interpretation of results and manuscript preparation. K.S.: experimental procedures, analyses and interpretation of results. M.C.C.: experimental procedures. C.A.A.: manuscript preparation and revision. J.A.: statistical analysis, participation in manuscript preparation and revision. J.D.: experimental design and manuscript revision. M.-M.D.: interpretation of results and manuscript revision.

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Conflict of interest

The authors have no conflict of interest to declare.

References

- Abir R, Feinmesser M, Yaniv I, Fisch B, Cohen JJ, Ben-Haroush A, Meirow D, Felz C, Avigad S. Occasional involvement of the ovary in Ewing sarcoma. *Hum Reprod* 2010;**25**:1708–1712.
- Abir R, Aviram A, Feinmesser M, Stein J, Yaniv I, Parnes D, Ben-Haroush A, Meirow D, Rabizadeh E, Fisch B. Ovarian minimal residual disease in chronic myeloid leukaemia. *Reprod Biomed Online* 2014;**28**:255–260.
- Amiot C, Angelot-Delettre F, Zver T, Alvergnas-Vieille M, Saas P, Garnache-Ottou F, Roux C. Minimal residual disease detection of leukemic cells in ovarian cortex by eight-color flow cytometry. *Hum Reprod* 2013;**28**:2157–2167.
- Amorim CA, Van Langendonck A, David A, Dolmans MM, Donnez J. Survival of human pre-antral follicles after cryopreservation of ovarian tissue, follicular isolation and in vitro culture in a calcium alginate matrix. *Hum Reprod* 2009;**24**:92–99.
- Anderson RA, Wallace WH. Antimüllerian hormone, the assessment of the ovarian reserve, and the reproductive outcome of the young patient with cancer. *Fertil Steril* 2013;**99**:1469–1475.
- Bastings L, Beerendonk CC, Westphal JR, Massuger LF, Kaal SE, van Leeuwen FE, Braat DD, Peek R. Autotransplantation of cryopreserved ovarian tissue in cancer survivors and the risk of reintroducing malignancy: a systematic review. *Hum Reprod Update* 2013;**19**:483–506.
- Brown H, Prescott R. *Applied Mixed Models in Medicine*, 2nd edn. West Sussex, England: John Wiley & sons Ltd, 2006.
- Czernobilsky B, Moll R, Levy R, Franke WW. Co-expression of cytokeratin and vimentin filaments in mesothelial, granulosa and rete ovarii cells of the human ovary. *Eur J Cell Biol* 1985;**37**:175–190.
- Dath C, Dethy A, Van Langendonck A, Van Eyck AS, Amorim CA, Luyckx V, Donnez J, Dolmans MM. Endothelial cells are essential for ovarian stromal tissue restructuring after xenotransplantation of isolated ovarian stromal cells. *Hum Reprod* 2011;**26**:1431–1439.
- de Lambert G, Poirot C, Guerin F, Brugieres L, Martelli H. Preservation of fertility in children with cancer. *Bull Cancer* 2015 (in press).
- De Vos M, Smits J, Woodruff TK. Fertility preservation in women with cancer. *Lancet* 2014;**384**:1302–1310.
- Dolmans MM, Martinez-Madrid B, Gadisseux E, Guiot Y, Yuan WY, Torre A, Camboni A, Van Langendonck A, Donnez J. Short-term transplantation of isolated human ovarian follicles and cortical tissue into nude mice. *Reproduction* 2007;**134**:253–262.
- Dolmans MM, Yuan WY, Camboni A, Torre A, Van Langendonck A, Martinez-Madrid B, Donnez J. Development of antral follicles after xenografting of isolated small human preantral follicles. *Reprod Biomed Online* 2008;**16**:705–711.
- Dolmans MM, Marinescu C, Saussoy P, Van Langendonck A, Amorim C, Donnez J. Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. *Blood* 2010;**116**:2908–2914.
- Dolmans MM, Luyckx V, Donnez J, Andersen CY, Greve T. Risk of transferring malignant cells with transplanted frozen-thawed ovarian tissue. *Fertil Steril* 2013;**99**:1514–1522.
- Donnez J, Dolmans MM. Fertility preservation in women. *Nat Rev Endocrinol* 2013;**9**:735–749.
- Donnez J, Dolmans MM. Transplantation of ovarian tissue. *Best Pract Res Clin Obstet Gynaecol* 2014;**28**:1188–1197.
- Donnez J, Martinez-Madrid B, Jadoul P, Van Langendonck A, Demille D, Dolmans MM. Ovarian tissue cryopreservation and transplantation: a review. *Hum Reprod Update* 2006;**12**:519–535.
- Donnez J, Dolmans MM, Pellicer A, Diaz-Garcia C, Sanchez Serrano M, Schmidt KT, Ernst E, Luyckx V, Andersen CY. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. *Fertil Steril* 2013;**99**:1503–1513.

- Donnez J, Dolmans M-M, Pellicer A, Diaz-Garcia C, Ernst E, Macklon KT, Andersen CY. Fertility preservation for age-related fertility decline. *Lancet* 2015;**385**:506–507.
- Edwards BK, Noone A-M, Mariotto AB, Simard EP, Boscoe FP, Henley SJ, Jemal A, Cho H, Anderson RN, Kohler BA et al. Annual report to the nation on the status of cancer, 1975–2010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast, or prostate cancer. *Cancer* 2014;**120**:1290–1314.
- Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod* 1996;**54**:197–207.
- Fasano G, Moffa F, Dechene J, Englert Y, Demeestere I. Vitrification of in vitro matured oocytes collected from antral follicles at the time of ovarian tissue cryopreservation. *Reprod Biol Endocrinol* 2011;**9**:150.
- Jin S, Lei L, Shea LD, Zelinski MB, Stouffer RL, Woodruff TK. Markers of growth and development in primate primordial follicles are preserved after slow cryopreservation. *Fertil Steril* 2010;**93**:2627–2632.
- Keros V, Xella S, Hultenby K, Pettersson K, Sheikhi M, Volpe A, Hreinsson J, Hovatta O. Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue. *Hum Reprod* 2009;**24**:1670–1683.
- Kim SS, Donnez J, Barri P, Pellicer A, Patrizio P, Rosenwaks Z, Nagy P, Falcone T, Andersen C, Hovatta O et al. Recommendations for fertility preservation in patients with lymphoma, leukemia, and breast cancer. *J Assist Reprod Genet* 2012;**29**:465–468.
- Knight PG, Glistler C. TGF-beta superfamily members and ovarian follicle development. *Reproduction* 2006;**132**:191–206.
- Lee WS, Yoon SJ, Yoon TK, Cha KY, Lee SH, Shimasaki S, Lee S, Lee KA. Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. *Mol Reprod Develop* 2004;**69**:159–163.
- Luyckx V, Dolmans MM, Vanacker J, Scalercio SR, Donnez J, Amorim CA. First step in developing a 3D biodegradable fibrin scaffold for an artificial ovary. *J Ovarian Res* 2013a;**6**:83.
- Luyckx V, Durant JF, Camboni A, Gilliaux S, Amorim CA, Van Langendonck A, Irenge LM, Gala JL, Donnez J, Dolmans MM. Is transplantation of cryopreserved ovarian tissue from patients with advanced-stage breast cancer safe? A pilot study. *J Assist Reprod Genet* 2013b;**30**:1289–1299.
- Luyckx V, Dolmans MM, Vanacker J, Legat C, Fortuno Moya C, Donnez J, Amorim CA. A new step toward the artificial ovary: survival and proliferation of isolated murine follicles after autologous transplantation in a fibrin scaffold. *Fertil Steril* 2014;**101**:1149–1156.
- Magoffin DA. Ovarian theca cell. *Int J Biochem Cell Biol* 2005;**37**:1344–1349.
- Magoffin DA, Magarelli PC. Preantral follicles stimulate luteinizing hormone independent differentiation of ovarian theca-interstitial cells by an intrafollicular paracrine mechanism. *Endocrine* 1995;**3**:107–112.
- Marcello MF, Nuciforo G, Romeo R, Di Dino G, Russo I, Russo A, Palumbo G, Schiliro G. Structural and ultrastructural study of the ovary in childhood leukemia after successful treatment. *Cancer* 1990;**66**:2099–2104.
- Martinez-Madrid B, Dolmans MM, Langendonck AV, Defrere S, Van Eyck AS, Donnez J. Ficoll density gradient method for recovery of isolated human ovarian primordial follicles. *Fertil Steril* 2004;**82**:1648–1653.
- Martinez-Madrid B, Camboni A, Dolmans MM, Nottola S, Van Langendonck A, Donnez J. Apoptosis and ultrastructural assessment after cryopreservation of whole human ovaries with their vascular pedicle. *Fertil Steril* 2007;**87**:1153–1165.
- Meirow D, Nugent D. The effects of radiotherapy and chemotherapy on female reproduction. *Hum Reprod Update* 2001;**7**:535–543.
- Meirow D, Dor J, Kaufman B, Shrim A, Rabinovici J, Schiff E, Raanani H, Levron J, Fridman E. Cortical fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury. *Hum Reprod* 2007;**22**:1626–1633.
- Meirow D, Hardan I, Dor J, Fridman E, Elizur S, Ra'anani H, Slyusarevsky E, Amariglio N, Schiff E, Rechavi G et al. Searching for evidence of disease and malignant cell contamination in ovarian tissue stored from hematologic cancer patients. *Hum Reprod* 2008;**23**:1007–1013.
- Navarini H, Reeves G. Specific stroma in the cortex and medulla of the ovary. 3. Changes induced by ionizing radiation. *Obstet Gynecol* 1972;**39**:556–561.
- Nilsson EE, Skinner MK. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biol Reprod* 2003;**69**:1265–1272.
- O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod* 2003;**68**:1682–1686.
- Orisaka M, Tajima K, Mizutani T, Miyamoto K, Tsang BK, Fukuda S, Yoshida Y, Kotsuji F. Granulosa cells promote differentiation of cortical stromal cells into theca cells in the bovine ovary. *Biol Reprod* 2006;**75**:734–740.
- Prasath EB, Chan ML, Wong WH, Lim CJ, Tharmalingam MD, Hendricks M, Loh SF, Chia YN. First pregnancy and live birth resulting from cryopreserved embryos obtained from in vitro matured oocytes after oophorectomy in an ovarian cancer patient. *Hum Reprod* 2014;**29**:276–278.
- Revel A, Revel-Vilk S, Aizenman E, Porat-Katz A, Safran A, Ben-Meir A, Weintraub M, Shapira M, Achache H, Laufer N. At what age can human oocytes be obtained? *Fertil Steril* 2009;**92**:458–463.
- Rosendahl M, Andersen MT, Ralfkiaer E, Kjeldsen L, Andersen MK, Andersen CY. Evidence of residual disease in cryopreserved ovarian cortex from female patients with leukemia. *Fertil Steril* 2010;**94**:2186–2190.
- Rosendahl M, Greve T, Andersen CY. The safety of transplanting cryopreserved ovarian tissue in cancer patients: a review of the literature. *J Assist Reprod Genet* 2013;**30**:11–24.
- Sanfilippo S, Canis M, Romero S, Sion B, Déchelotte P, Pouly JL, Janny L, Smitz J, Brugnion F. Quality and functionality of human ovarian tissue after cryopreservation using an original slow freezing procedure. *J Assist Reprod Genet* 2013;**30**:25–34.
- Shea LD, Woodruff TK, Shikanov A. Bioengineering the ovarian follicle microenvironment. *Annu Rev Biomed Eng* 2014;**16**:29–52.
- Spears N, Boland NI, Murray AA, Gosden RG. Mouse oocytes derived from in vitro grown primary ovarian follicles are fertile. *Hum Reprod* 1994;**9**:527–532.
- Stouffer RL, Martinez-Chequer JC, Molskness TA, Xu F, Hazzard TM. Regulation and action of angiogenic factors in the primate ovary. *Arch Med Res* 2001;**32**:567–575.
- Telfer EE, McLaughlin M. In vitro development of ovarian follicles. *Semin Reprod Med* 2011;**29**:15–23.
- Telfer EE, Zelinski MB. Ovarian follicle culture: advances and challenges for human and nonhuman primates. *Fertil Steril* 2013;**99**:1523–1533.
- Telfer EE, McLaughlin M, Ding C, Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod* 2008;**23**:1151–1158.
- Vanacker J, Camboni A, Dath C, Van Langendonck A, Dolmans MM, Donnez J, Amorim CA. Enzymatic isolation of human primordial and primary ovarian follicles with Liberase DH: protocol for application in a clinical setting. *Fertil Steril* 2011;**96**:379–383 e373.
- Vanacker J, Luyckx V, Dolmans MM, Des Rieux A, Jaeger J, Van Langendonck A, Donnez J, Amorim CA. Transplantation of an alginate-matrigel matrix containing isolated ovarian cells: first step in developing a biodegradable scaffold to transplant isolated preantral follicles and ovarian cells. *Biomaterials* 2012;**33**:6079–6085.
- Wallace WH, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: who is at risk and what can be offered? *Lancet Oncol* 2005;**6**:209–218.