

Endothelial cells are essential for ovarian stromal tissue restructuring after xenotransplantation of isolated ovarian stromal cells

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BACKGROUND: Grafting of isolated follicles represents an approach to prevent the risk of reimplanting malignant cells with cryopreserved ovarian fragments. Optimal conditions and cell types required to sustain human follicular growth need to be identified. To help improve the grafting technique, we investigated whether short-term xenografting of a suspension containing ovarian stromal and endothelial cells without follicles could enhance graft survival and revascularization.

METHODS: In human ovary, CD34 selectively labels endothelial cells of blood vessels. A CD34-replete ovarian stromal cell group, including stromal and endothelial cells, was obtained after enzymatic digestion of fresh human ovarian cortex. Magnetic-activated cell sorting was used to establish a CD34-depleted ovarian stromal cell group. Proportions of CD34-positive cells were evaluated by flow cytometry and immunocytochemistry. Cell suspensions were embedded in human plasma clots and grafted ($n = 10$ for each group, 7 days) to the ovarian bursa of nude mice. Angiogenesis was quantified after human/mouse CD34 immunostaining.

RESULTS: CD34-replete grafts had a well-organized and vascularized stromal structure, containing tubular components staining for human CD34 and corresponding to functional vessels, as evidenced by intraluminal red blood cells. CD34-depleted grafts tended to be smaller than CD34-replete grafts and poorly vascularized with central necrosis. Global microvessel density was higher in the CD34-replete than depleted group (337.9 versus 187.3 vessels/mm², $P < 0.05$), with a greater proportion of human vessels (68.02 versus 6.95%, respectively, $P < 0.05$).

CONCLUSIONS: We demonstrated the importance of co-transplanting ovarian endothelial and stromal cells to ensure the formation of a well-vascularized and structured ovarian-like stroma after short-term xenografting, for future application in the transplantation of isolated follicles.

Key words: ovarian tissue / endothelial cells / ovarian stromal cells / xenotransplantation / magnetic-activated cell sorting

Introduction

Cryopreservation and transplantation of ovarian tissue is the only option for fertility preservation currently available for prepubertal children and young patients at risk of premature ovarian failure, who require immediate chemo- and/or radiotherapy (Donnez *et al.*, 2006, 2008). To date, 13 live births have been achieved worldwide after autologous transplantation of cryopreserved ovarian cortex (Donnez *et al.*, 2004; Meirow *et al.*, 2005; Demeestere *et al.*, 2007; Andersen *et al.*, 2008; Silber *et al.*, 2008; Donnez *et al.*, 2010; Roux *et al.*, 2010; Sánchez-Serrano *et al.*, 2010). Despite these encouraging results, this technique still needs to be

optimized. The period of hypoxia following avascular grafting appears to be one of the main obstacles to successful transplantation of ovarian tissue (Nugent *et al.*, 1998; Yang *et al.*, 2008; Demeestere *et al.*, 2009).

On the other hand, new options need to be developed for patients at risk of transfer of malignant cells. Indeed, the presence of malignant cells in ovarian tissue cannot be excluded and may lead to recurrence of the primary disease after reimplantation of frozen-thawed ovarian fragments (Meirow *et al.*, 2008). In a very recent paper, Dolmans *et al.* (2010) proved ovarian contamination by malignant cells by quantitative RT-PCR analysis and long-term xenografting of ovarian cortex from patients with acute or chronic leukemia.

Besides *in vitro* culture, grafting of isolated primordial follicles could represent an interesting alternative to prevent the risk of reimplanting malignant cells (Dolmans et al., 2007, 2008; Smitz et al., 2010). By xenografting the isolated human follicles in a plasma clot, it was shown that follicles are able to survive and grow after short- and long-term grafting (Dolmans et al., 2007, 2008). Histological analysis of the grafts after 7 days and 5 months demonstrated the presence of well-organized and vascularized stroma-like tissue of human origin that is able to support the development of grafted isolated follicles. However, only 20% of follicles were recovered after short-term xenografting (Dolmans et al., 2007). Several factors may explain this massive follicular loss after transplantation. One of the most important factors is the delay that occurs in the re-establishment of vascularization during the first days following grafting (Van Eyck et al., 2009). Furthermore, follicles need to maintain their three-dimensional structure to grow, and lack of tissue support after grafting could also induce this follicular loss (Amorim et al., 2009). Results from Gosden's study of enzymatically digested ovarian tissue autotransplanted into the ovarian bursa of mice showed that the grafted suspension reorganized into a functioning organ (Gosden, 1990) and resulted in restoration of fertility after transplantation (Carroll and Gosden, 1993).

These findings suggest that other ovarian cell types, such as stromal and endothelial cells, are essential to ensure graft survival and quality. As described in other xenografting models, like pancreatic islets, endothelial cells appear to play a crucial role in graft survival by improving the quality of revascularization after grafting (Mendola et al., 1994).

This led us to propose a new strategy for xenografting the isolated follicles. Indeed, if stromal and endothelial cells are needed to sustain follicular growth, fully isolated follicles from cryopreserved ovarian tissue could be grafted together with autologous fresh stromal and endothelial cells from a new ovarian biopsy after cancer treatment. This clot/xenotransplantation model could then be used to study the interactions between follicles, stromal cells and endothelial cells, allowing appropriate control of graft composition.

To our knowledge, there are no data available on optimal conditions or cell types required to sustain human follicular growth after grafting of isolated follicles, with a view to improving recovery and survival rates. This study was designed to evaluate whether the presence of endothelial cells would enhance survival of fresh isolated human ovarian stromal cells, as well as revascularization, after short-term xenografting. For this purpose, the clot xenotransplantation model was applied to compare grafting of human ovarian stromal cells, either replete with, or depleted of, endothelial cells, for 7 days in a murine model. In order to analyze interactions only between ovarian stromal cells and endothelial cells, as well as graft survival, isolated follicles were not included in grafted suspensions. A procedure to isolate human ovarian stromal cells was set up using enzymatic digestion by collagenase, and magnetic-activated cell sorting (MACS) was developed as a strategy to deplete suspensions of endothelial cells.

Materials and Methods

Experimental design

The experimental design of the study is shown in Fig. 1.

The CD34 antigen is a single-chain transmembrane glycoprotein of 105–120 kDa expressed on human hematopoietic progenitor cells,

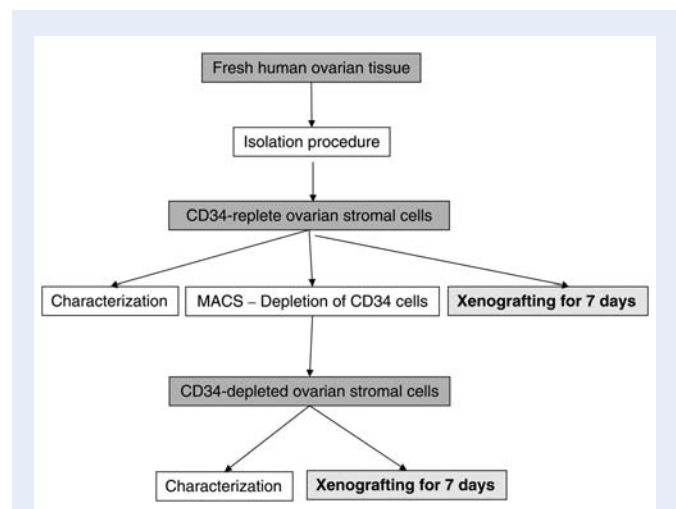


Figure 1 Experimental design. The CD34-replete human ovarian stromal cell group was obtained after enzymatic digestion. The CD34-depleted human ovarian stromal cell group was obtained after depletion of CD34-positive cells by MACS. The two cell groups were characterized; cells were embedded in plasma clots and then xenografted for 7 days in nude mice.

endothelial progenitor cells and vascular endothelial cells. In the human ovary, CD34 selectively labels endothelium of either newly formed blood vessels (in the theca layer of early antral follicles or the granulosa-lutein layer of newly formed corpora lutea) or mature blood vessels, such as arteries deep in the ovarian cortex (Delgado-Rosas et al., 2009).

Fresh human ovarian cortex was used for enzymatic digestion. The suspension obtained was termed CD34-replete ovarian stromal cells and divided into three parts. One part underwent a characterization procedure, another was depleted of CD34 cells by MACS and the last part was embedded in a human plasma clot and grafted for 7 days to the ovarian bursa of nude mice. The suspension obtained after depletion was termed CD34-depleted ovarian stromal cells and this group was characterized and grafted in the same conditions as the CD34-replete group. Characterization of cells was carried out in all cases. Grafting was performed when enough tissue was available ($n = 10$).

Collection of ovarian tissue

Use of human ovarian tissue was approved by the Institutional Review Board of the Université Catholique de Louvain. Ovarian biopsies of approximately 20×10 mm were obtained from patients between 42 and 78 years of age ($50.8 \text{ years} \pm 6.42$, mean $\pm$ SD) undergoing laparoscopic hysterectomy for benign gynecologic disease. Ovarian tissue from peri-menopausal and menopausal patients was used because the vascular network of ovarian cortex from these women is similar to that of ovaries exposed to chemotherapy (Delgado-Rosas et al., 2009). Biopsies were immediately transported on ice to the laboratory in Dulbecco's modified Eagle's medium (DMEM)-F12 medium containing L-glutamine and 15 mM HEPES (Gibco, Invitrogen, Merelbeke, Belgium).

Isolation procedure

The medullar part of the biopsy was removed with surgical scissors, and the cortical tissue was cut into uniform-size pieces of $0.5 \times 0.5 \times 1$ mm using a tissue sectioner (McIlwain Tissue Chopper, Mickel Laboratory, Guildford, UK), adjusted to 0.5 mm. Minced human ovarian tissue was incubated in 10 ml DMEM-F12 medium (Invitrogen) supplemented with

1 mg/ml of collagenase IA (C2674, Sigma-Aldrich, Bornem, Belgium). Incubation was performed in a water bath at 37°C for 35 min. The tissue was pipetted every 10 min to mechanically disrupt digested tissue. Enzymatic activity was inactivated by adding an equal volume of cold DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich). The digested cell solution was successively filtered through sterilized 80 and 11 µm nylon net filters (Millipore, Brussels, Belgium). After centrifugation, the pellet was re-suspended in 2 ml of DMEM-F12 + 10% FBS medium, and cells were counted and divided into two groups. The first group, consisting of CD34-replete ovarian stromal cells, was used for characterization of cells and grafting. The second group, consisting of CD34-depleted ovarian stromal cells, first underwent depletion of CD34 cells by MACS and was then characterized before grafting.

Depletion of CD34 cells by MACS

Dissociated cells were re-suspended in 300 µl of phosphate-buffered saline (PBS without Ca and Mg, Lonza, Braine-l'Alleud, Belgium) and supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA (MACS buffer, following the manufacturer's instructions) per 10⁸ cells. One hundred microliters of FcR blocking reagent per 10⁸ cells and 100 µl of CD34 microbeads per 10⁸ cells were then added to the suspension (130-046-702, Miltenyi Biotec, Utrecht, the Netherlands). After mixing, the cells were incubated for 30 min at 4°C, before being washed with excess MACS buffer and re-suspended in 500 µl buffer per 10⁸ cells. To deplete the suspension of CD34-positive cells, MACS was carried out. Magnetic separation of cells was performed using an MS column (Miltenyi Biotec) placed in a MiniMACS separator (Miltenyi Biotec), according to the manufacturer's protocol. The depleted solution was then characterized by flow cytometry (FC) and immunocytochemistry (ICC) or embedded in a plasma clot for grafting.

Characterization of isolated and sorted cells by FC and ICC

The percentage of CD34-positive cells was evaluated in CD34-replete and CD34-depleted groups by FC and ICC. Analysis was also performed to evaluate the percentage of vimentin-positive cells by ICC. Vimentin is a cytoskeleton protein mainly expressed in the cytoplasm of cells of mesenchymal origin, and strongly expressed in ovarian stromal connective tissue (Czernobilsky *et al.*, 1985).

For FC analysis, cells were suspended at a concentration of 10⁶ cells/ml in PBS with 0.5% BSA, and then incubated in the dark at 4°C with monoclonal human anti-CD34 antibody conjugated to R-phycoerythrin (R-PE) (1/10 dilution, ref. 130-081-002, Miltenyi Biotec). Negative controls included cells incubated with immunoglobulins of the corresponding isotype coupled to R-PE (1/10 dilution, BD Biosciences, San Jose, CA, USA). After 20 min, cells were washed and re-suspended in 300 µl of CellFIX solution (BD Biosciences). They were then analyzed on a FACS-Canto II Flow Cytometer (BD Biosciences), and at least 10 000 events were acquired for each sample. Results were expressed as the percentage of positive cells in the suspension.

For ICC, cells were fixed in 4% formaldehyde for 1 h, and cytospin slides were prepared using the Thermo Electron Cytospin 2. For each experiment, one slide was stained with hematoxylin and eosin (H&E) (Merck, Darmstadt, Germany) for histological analysis and the rest was assigned to immunostaining (Superfrost Plus slides, Menzel-Glaser, Braunschweig, Germany). The slides were first rehydrated and permeabilized by immersion in Tris-buffered saline (TBS 0.05M, pH 7.4) supplemented with 0.05% Triton X-100 (Merck). A previously described protocol was applied for immunostaining (Dolmans *et al.*, 2007) using the following primary antibodies: mouse anti-human CD34 (1/500,

clone QBend/10, Biocare Medical, Mechelen, Belgium) and mouse anti-human vimentin (1/100, clone V9, Dako, Heverlee, Belgium), incubated overnight at 4°C. Human umbilical vein endothelial cells (donated by the Angiogenesis and Cancer Research Group, Pole of Pharmacology and Therapeutics, Université Catholique de Louvain, Brussels, Belgium) and cultured stromal cells were used as positive controls for CD34 and vimentin labeling, respectively. Negative controls included slides incubated with universal negative control mouse immunoglobulin (Ig) (Dako). Slides were examined blindly by two independent observers Catherine Dath (CD) and Alice Dethy (AD). The percentage of positive cells was evaluated under a microscope (Zeiss, Munich, Germany) at ×400 magnification by counting a minimum of 200 cells/slide. As no difference in results was observed between the two observers, results were averaged.

Transplantation to nude mice

Biopsies for grafting came from 10 patients. After cell isolation, one part of the suspension was grafted directly and the other part underwent depletion of CD-34-positive cells prior to transplantation. Cells were embedded in plasma clots, which served as a vehicle to facilitate subsequent grafting, as previously described by Gosden (1990) and Dolmans *et al.* (2007). To obtain a clot, the patient's blood was centrifuged at 405g for 15 min at 4°C and the supernatant plasma was recovered. Under a stereo-microscope, 5 µl of suspension containing either 50 000 CD34-replete ovarian stromal cells or 50 000 CD34-depleted ovarian stromal cells was injected into a 20 µl droplet of human autologous venous plasma. Clotting was induced by adding 20 µl of 0.025 M CaCl₂, followed by incubation at 37°C for 30 min. The clot then acquired a jelly-like consistency and it was possible to pick it up with surgical forceps to transplant it into the murine ovarian bursa.

The guidelines for animal welfare were approved by the Committee on Animal Research of the Université Catholique de Louvain. Twenty-seven-week old Swiss nu/nu female mice (Charles River B Laboratories, Brussels, Belgium) were used for the study and bred as previously described (Nisolle *et al.*, 2000). The mice were anesthetized according to an existing protocol (Dath *et al.*, 2010). A medial dorsal cutaneous incision was made and the peritoneum was opened through a small hole. The periovarian fat was gently gripped with forceps and exposed to give access to the ovarian bursa. Under operative microscopic control (Leica MZ6, Wetzlar, Germany), a small slit was made in the ovarian bursal membrane to introduce a CD34-replete or -depleted clot. The adnexa were replaced into the peritoneal cavity without stitching the peritoneum and the same procedure was performed on the contralateral side. Finally, the skin was closed with EZ clips (9 mm, Stoelting Co., Wood Dale, IL, USA) and anesthesia was reversed by injection of atipamezole (1 mg/kg; Antisedan, Pfizer, Louvain-la-Neuve, Belgium). The animals were euthanized after 7 days by inhalation of carbon monoxide and all murine ovarian bursae were recovered and fixed in 4% formaldehyde.

Analysis of recovered grafts by histology and immunohistochemistry

Each graft, having been formalin-fixed and paraffin-embedded, was cut into serial sections of 5 µm. Every sixth section was stained with H&E for histological analysis and the others were assigned to immunostaining. The human origin of the cells and proliferation in the clots were confirmed by anti-human vimentin and anti-human Ki67 immunostaining, respectively, according to a previously described protocol (Dolmans *et al.*, 2007). Human and murine vessels were assessed by double CD34 immunostaining. Sections were deparaffinized with Histosafe (Yvsolab SA, Beerse, Belgium) and rehydrated in 2-propanol (Merck). After blocking

endogenous peroxidase activity, sections were incubated for 30 min with goat serum to block non-specific binding sites before incubation with rat anti-mouse CD34 antibody (1:100, clone MEC 14.7, Hycult Biotech, Uden, the Netherlands) for 1 h at room temperature. The slides were then incubated for 30 min at room temperature with rabbit anti-rat IgG biotinylated antibody (1:100, BA 4001, Vector Laboratories, Peterborough, UK) and then with streptavidin for 30 min at room temperature (1:500, P0397, Dako). Diaminobenzidine was used as a chromogen. Non-specific binding sites were again blocked for 30 min before overnight incubation with mouse anti-human CD34 antibody (1:500) at 4°C. Thereafter, the slides were incubated for 60 min at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG (1:300, Jackson ImmunoResearch, Suffolk, UK), and Fast-Red/Naphthol AS-MX (F4648, Sigma-Aldrich) was used as a chromogen. Slides were then counterstained with hematoxylin and mounted with aqueous mounting medium (Aquatex, Merck). Negative controls consisted of rat and mouse IgGs (Sigma-Aldrich) diluted to the same concentrations as the primary antibodies. The species specificity of each antibody was confirmed using human and mouse ovaries as appropriate positive controls.

Quantitative analysis of angiogenesis in recovered grafts

After double CD34 immunostaining, slides were digitized with the Mirax Scan system (Carl Zeiss AG, Germany) and morphometric analysis was applied to count the vessels and delimit surface areas. Angiogenesis in the recovered grafts was evaluated by microvessel density (MVD), defined as the number of microvessels per millimeter squared, and by endothelial area (EA), defined as the percentage of fragment surface area occupied by endothelial cells. The MVD and EA were assessed in both human (hMVD, hEA) and murine (mMVD, mEA) vessels. Three different sections randomly distributed in the grafted clots were analyzed.

Statistical analysis

Analyses were performed using the PASW Statistics 18 program (Chicago, IL, USA) for Windows. A paired sample *t*-test was conducted to validate the double-blind procedure, and results obtained by ICC and FC were analyzed by an independent sample *t*-test in order to compare the techniques. The same test was performed to compare results obtained by ICC in the CD34-replete group and the CD34-depleted group. For FC analysis, the same patient was evaluated in both groups and a paired sample *t*-test was applied. Morphometric results were analyzed by the Mann–Whitney *U*-test for non-parametric independent two-group comparisons. Values of at least $P < 0.05$ were considered statistically significant.

Results

Characterization of cells after repletion and depletion

Efficiency of the depletion method

The proportion of CD34-positive cells was assessed in isolated cell suspensions (CD34-replete group) and in cell suspensions obtained after depletion of CD34 cells (CD34-depleted group), in order to evaluate the efficiency of depletion. After depletion by MACS, the percentage of CD34-positive cells was significantly lower than in the CD34-replete group ($P < 0.05$), as assessed by FC and ICC (Table I). As an additional analysis to confirm the efficiency of depletion, the percentage of vimentin-positive cells was evaluated by ICC. This percentage was found to have increased after depletion of CD34 cells ($P < 0.05$) (Table I).

Comparison of characterization techniques

FC and ICC were applied to evaluate the percentage of CD34-positive cells in CD34-replete and CD34-depleted groups. After isolation and filtration, the percentage of CD34-positive cells identified in CD34-replete suspensions was 18.04% by FC and 14.04% by ICC (Table I). After depletion by MACS, the percentage of CD34-positive cells identified in CD34-depleted suspensions was 1.05% by FC and 0.64% by ICC. No difference was observed between the two techniques ($P = 0.284$ and $P = 0.267$ in the CD34-replete and CD34-depleted groups, respectively).

Analysis of the recovered grafts

Graft recovery rate

Seven days after transplantation, grafted clots were removed and identified by histological analysis and specific anti-human vimentin immunostaining. Seventy percent (7/10) of the grafts were clearly identified by histology in the CD34-replete group and 60% (6/10) in the CD34-depleted group. This loss can be explained by the extrusion of some grafts out of the ovarian bursa after the grafting procedure.

Graft size (Fig. 2)

The size of recovered grafts was measured by calculating their volume on digitized slides (Mirax Scan) after the morphometric analysis. The mean volumes of CD34-replete and CD34-depleted grafts were 0.82 ± 0.53 and $0.33 \pm 0.17 \text{ mm}^3$, respectively ($P < 0.063$). Figure 2 illustrates the histological aspect of both types of grafts.

Table I Efficiency of the depletion method.

Group	CD34-positive cells (% $\pm$ SD)		Vimentin-positive cells (% $\pm$ SD)
	FC	ICC	ICC
CD34-replete group $n = 14$	$18.04^a \pm 11.27$	$14.04^a \pm 6.78$	$74.07^a \pm 5.27$
CD34-depleted group $n = 10$	$1.05^b \pm 0.91$	$0.64^b \pm 0.57$	$85.36^b \pm 4.06$

Control (CD34-replete) and CD34-depleted human ovarian stromal cells were analyzed by flow cytometry (FC) and immunocytochemistry (ICC).

^aIs significantly different from ^b($P < 0.05$) within columns.

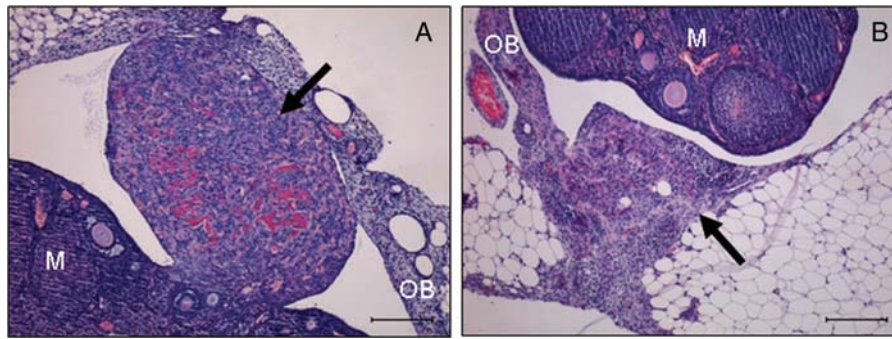


Figure 2 The size of recovered grafts. H&E staining of a CD34-replete graft (**A**, $n = 1$) and a CD34-depleted graft (**B**, $n = 1$), illustrating the difference in graft size observed between the two groups. The same number of cells was grafted for 7 days inside a clot placed in the murine ovarian bursa. The black arrow indicates the localization of the grafted clot (scale bar = 200 μm). M, murine ovary; OB, ovarian bursa. Photomicrographs are representative of all replete and depleted recovered grafts.

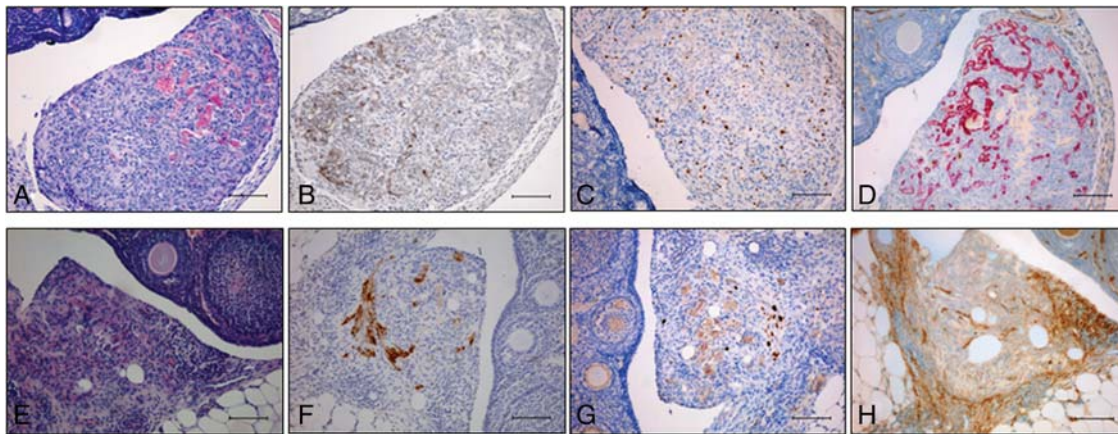


Figure 3 Histological and immunohistochemical analysis of the grafts. The four figures show different immunostaining on the same graft specimen. (**A–D**) show CD34-replete ovarian stromal cells inside a clot grafted to the murine ovarian bursa for 7 days ($n = 1$). (**E–H**) show CD34-depleted ovarian stromal cells grafted in the same conditions ($n = 1$). (**A** and **E**) H&E staining. (**B** and **F**) Specific anti-human vimentin staining (positive cells in brown). (**C** and **G**) Specific anti-human Ki-67 immunostaining (positive cells in brown). (**D** and **H**) Double CD34 immunostaining (human vessels in red, murine vessels in brown). Scale bar = 100 μm . Photomicrographs are representative of all replete and depleted recovered grafts.

Histological and immunohistochemical analysis of the grafts (Figs 3 and 4)

In the CD34-replete group, a well-organized and well-vascularized stromal structure was clearly observed in the murine ovarian bursa (Fig. 3A). This ovarian-like stroma showed tissue composed of densely packed spindle-shaped, fibroblast-like cells, similar to that of the native ovary. Specific anti-human vimentin immunostaining confirmed a predominance of human cells in the grafted clots, and their proliferative activity was evidenced by positive Ki-67 immunostaining (Fig. 3B and C). After double CD34 immunostaining, tubular structures staining positive for human CD34 were observed in the grafts, corresponding to functional vessels, as demonstrated by the presence of intraluminal red blood cells (Fig. 4A). Murine vessels were predominant at the periphery of grafts, probably emerging from the capsule of the ovarian bursa. Human vessels were randomly distributed in both the center and the periphery of whole grafts. These human vessels

were found to coexist with vessels of murine origin in variable proportions. (Fig. 4B–D).

In the CD34-depleted group, all the grafted clots were more difficult to identify by histology and anti-human vimentin immunostaining was systematically needed to localize the grafts (Fig. 3F). In contrast to the CD34-replete group, grafts were small and poorly vascularized, and areas of central necrosis were frequently observed. Necrosis was characterized by the presence of pyknotic nuclei in stromal cells and signs of cytolysis, including the presence of cell debris and an empty cytoplasm. Vessels were almost exclusively of murine origin, as illustrated in Fig. 3H, and were predominantly found at the periphery of grafts.

Morphometric and quantitative analysis of the grafts (Fig. 5)

Seven days after grafting, global MVD was significantly higher in the CD34-replete group than the CD34-depleted group (337.9 ± 177.5

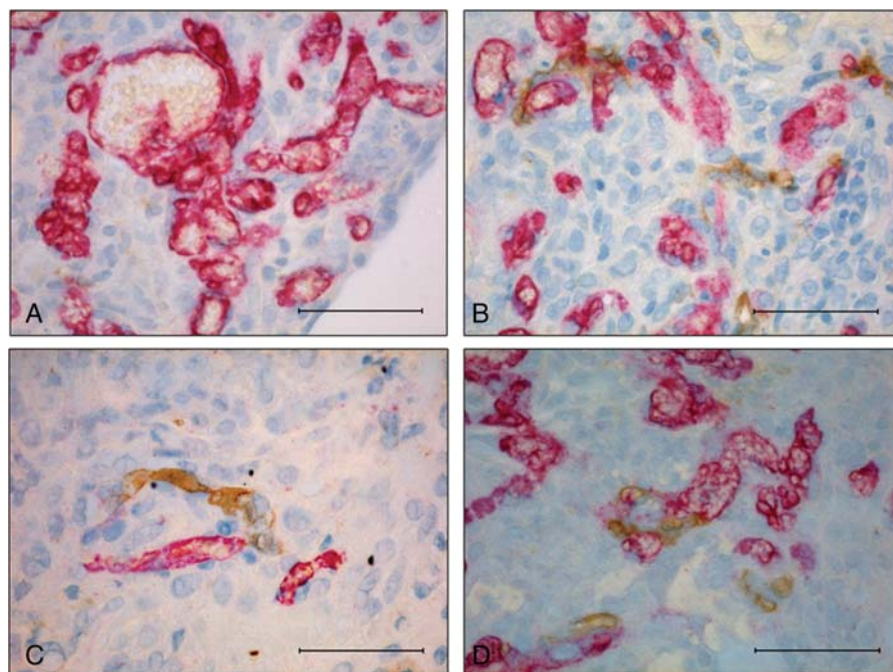


Figure 4 Histological and immunohistochemical analysis of the grafts. Double CD34 immunostaining of human vessels (red) and murine vessels (brown) in CD34-replete grafts. (A) Shows tubular structures staining positive for human CD34, whose functionality is evidenced by the presence of intraluminal red blood cells. (B–D) illustrate the presence of human and murine vessels coexisting in variable proportions in grafts ($n = 2$). Scale bar = 50 μm .

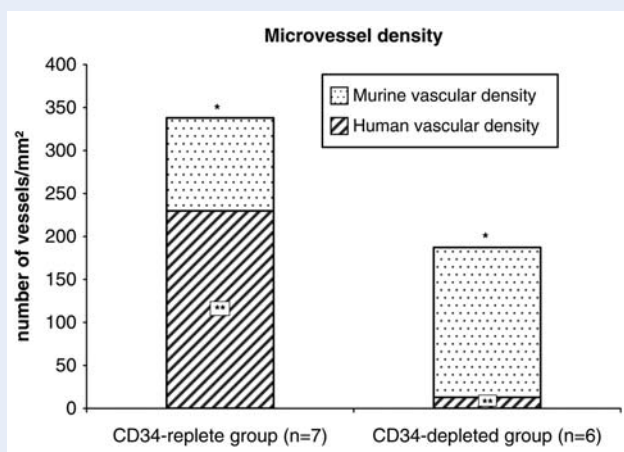


Figure 5 Morphometric and quantitative analysis of the grafts. MVD in CD34-replete ($n = 7$) and CD34-depleted grafts ($n = 6$). Seven days after grafting, global MVD was higher in the CD34-replete group than the CD34-depleted group (*) (337.9 ± 177.5 versus 187.3 ± 123.0 vessels/ mm^2 ; $P < 0.05$). In the CD34-replete group, human vascular density was higher than in the CD34-depleted group (**) ($P < 0.05$).

versus 187.3 ± 123.0 vessels/ mm^2 ; $P < 0.05$), as illustrated in Fig. 5. In CD34-replete grafts, a significantly higher proportion of vessels of human origin was observed compared with CD34-depleted grafts (68.02% versus 6.95%, $P < 0.05$). In CD34-depleted grafts, the vascular

network was mainly composed of vessels of murine origin, constituting 93.05% of global vascular density. A similar pattern was encountered for EA (data not shown). Global EA, corresponding to the percentage of fragment surface area occupied by endothelial cells, tended to be higher in CD34-replete grafts than CD34-depleted grafts (5.74 versus 2.44%, respectively, $P = 0.63$), as was hEA, found to be 4.32% in the CD34-replete group compared with 0.01% in the CD34-depleted group ($P < 0.05$).

Discussion

This is the first study to analyze revascularization occurring after xenografting of isolated ovarian cells. Some interesting studies have been published on the early revascularization process, but are related to xenotransplantation of human ovarian fragments. Indeed, Van Eyck et al. (2009, 2010) recently demonstrated that an initial period of hypoxia observed during the first 5 days after grafting was followed by progressive graft reoxygenation and reperfusion from day 5 onwards. Ischemic damage occurring during this initial post-transplantation period could induce primordial follicle loss (Aubard et al., 1999; Baird et al., 1999; Nisolle et al., 2000) and disordered follicular activation (Donnez et al., 2006; Dolmans et al., 2007; Schubert et al., 2008). In the superficial ovarian cortex, primordial and early growing follicles do not possess an independent vascular network and are therefore dependent on their proximity to stromal vessels (Delgado-Rosas et al., 2009).

As described for other tissues, endothelial cells appear to play a crucial role by improving the quality of revascularization after grafting.

In a study by Mendola *et al.* (1997), rats were auto-grafted either with vascularized whole pancreatic islets or with a suspension of purified pancreatic β -cells. Whole islets completed revascularization by days 3–5 after transplantation, compared with days 10–14 in purified β -cell grafts. This marked delay in revascularization was attributed to the lack of endothelial cells in the purified β -cell graft suspensions.

Our study was designed to compare xenografting of human ovarian stromal cells, either replete with or depleted of CD34-positive cells, in a murine model. CD34 is a transmembrane protein known to be expressed by mature and immature endothelial cells, hematopoietic progenitor cells and endothelial progenitor cells (Pusztaszeri *et al.*, 2006). Repletion of suspensions with CD34-positive cells was obtained by enzymatic digestion and filtration, while MACS was used to deplete suspensions of CD34 cells. In CD34-replete grafts, global vascular density was found to be significantly higher and mainly a result of the presence of human vessels. In CD34-depleted grafts, vascularization was significantly lower and almost entirely dependent on peripheral invasion by murine vessels.

The most striking phenomenon observed in our study was the formation of vascular tubular structures of human origin after xenografting of suspensions replete with CD34-positive cells. These vascular structures probably originate from the reorganization of grafted isolated human CD34-positive cells obtained after enzymatic digestion of ovarian cortex. The human origin of the cells was proved by use of human-specific antibodies for FC and ICC analyses. Two hypotheses may be proposed to explain the nature of these human CD34-positive cells. They could be differentiated endothelial cells, originating from ovarian stromal vessels. However, one cannot exclude the possible presence of endothelial progenitor cells in the ovarian cell suspension, which could contribute to vascular network formation.

The fact that isolated endothelial cells are able to reorganize into functional vessels has previously been studied in skin transplantation models. Human dermal microvascular endothelial cells transplanted on biodegradable polymer matrices were found to have dispersed throughout the sponge one day after transplantation, organized themselves into empty tubular structures by Day 5, and differentiated into functional microvessels within 7–10 days (Nör *et al.*, 2001). These results are consistent with our observation of microvessels in grafts 7 days after transplantation, proved to be functional by the presence of intraluminal red blood cells. This is the first time that reorganization of endothelial cells into functional vessels has been described in the ovary after transplantation of isolated ovarian cells.

In our study, CD34-replete grafts tended to be larger in size than CD34-depleted grafts, although the same number of cells was grafted in both instances. This difference may be explained by the phenomenon of early hypoxia, which could induce cell necrosis or activation of apoptotic pathways (Israely *et al.*, 2006; Yang *et al.*, 2008). Central necrosis was observed on histological sections of CD34-depleted grafts, but not CD34-replete grafts. Van der Strate *et al.* (2007) reported that human CD34-positive cells, implanted in a hypoxic environment, generate an angiogenic niche by secreting chemotactic and angiogenic factors, enabling rapid neovascularization. Indeed, isolated grafted cells remain sensitive to hypoxic stimuli, resulting in upregulation of hypoxia-inducible factor 1, overexpression of vascular endothelial growth factor and development of new blood vessels (Laschke *et al.*, 2009).

After 7 days of grafting, vascular density was found to be significantly higher in CD34-replete grafts. As proved by double CD34 immunostaining, both human and murine vessels were involved in the vascularization of grafts. The participation of both human and murine vessels in graft revascularization has previously been described for ovarian fragment xenotransplantation by Van Eyck *et al.* (2010).

In CD34-depleted grafts, vascular density was significantly lower. Owing to the lack of human endothelial cells in the grafted suspension, more than 90% of vessels in the graft were of murine origin. In this case, vascularization is almost exclusively dependent on peripheral invasion by host vessels. However, peripheral invasion of the graft by murine vessels does not appear to be sufficient to ensure survival of the whole graft after transplantation.

The advantages of having CD34-positive cells in the suspension prior to grafting are increased revascularization in both the center and periphery of the graft, and good survival of co-transplanted cells. Better survival of co-transplanted cells, associated with production of growth factors by endothelial cells, was also described in the field of bone tissue engineering (Grellier *et al.*, 2009).

By shortening the duration of ischemia, we could expect to reduce follicular degeneration reported after xenografting of isolated follicles (Dolmans *et al.*, 2007), and therefore maximize the surviving pool of follicles and improve the probability for subsequent successful maturation of oocytes (Israely *et al.*, 2006).

In conclusion, we set up a procedure to isolate human ovarian stromal cells using enzymatic digestion by collagenase, and using MACS as a strategy to deplete the suspension of endothelial cells. These procedures could be applied as validated tools to precisely control the proportion of CD34 cells in cell suspensions. Nevertheless, further studies are required to analyze the survival of isolated human ovarian follicles co-transplanted with endothelial and stromal cells. Our preliminary data show that human ovarian follicles grafted for 1 week with stromal and endothelial cells appear to be healthy and surrounded by restructured ovarian tissue (Supplementary data, Fig. S1A and B). In a clinical approach, fully isolated human follicles obtained from cryopreserved ovarian tissue could be grafted together with autologous fresh stromal and endothelial cells from a new ovarian biopsy after cancer treatment. The safety of this option will need to be ascertained but we believe that grafting fully isolated follicles together with cells obtained from disease-free ovarian tissue will be consistent with safe medical practice. As the plasma clot model used here may be inappropriate for clinical application because of technical constraints and potential toxicity of the plasma, it is vital to develop an artificial matrix for grafting of isolated pre-antral follicles.

In this study, we demonstrated the importance of co-transplanting ovarian endothelial cells together with stromal cells to ensure the formation of a well-vascularized and structured ovarian-like stroma, an essential component of an optimal scaffold to support survival and growth of grafted isolated follicles.

Authors' roles

C.D. played a role in study design, experimental procedures, analysis of results, statistical analysis and manuscript preparation; A.D. carried out experimental procedures; A.S.V.E. and C.A. contributed to the discussion; M.M.D. and A.V.L. took part in supervision, interpretation

of results and manuscript revision; J.D. was involved in manuscript revision and final approval of the version to be published.

Supplementary data

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

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