Isoform 111 of Vascular Endothelial Growth Factor ($VEGF_{111}$) Improves Angiogenesis of Ovarian Tissue Xenotransplantation

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Background. Cryopreservation of cortex ovarian tissue before anticancer therapy is a promising technique for fertility preservation mainly in children and young women. Ischemia in the early stage after ovarian graft causes massive follicle loss by apoptosis. $VEGF_{111}$ is a recently described vascular endothelial growth factor (VEGF) isoform that does not bind to the extracellular matrix, diffuses extensively, and is resistant to proteolysis. These properties confer a significantly higher angiogenic potential to $VEGF_{111}$ in comparison with the other VEGF isoforms.
Methods. We evaluated the morphology of cryopreserved sheep ovarian cortex grafted in the presence or absence of $VEGF_{111}$. Ovarian cortex biopsies were embedded in type I collagen with or without $VEGF_{111}$ addition before transplantation to severe combined immunodeficient mice ovaries. Transplants were retrieved 3 days or 3 weeks later. Follicular density, vasculature network, hemoglobin content, and cell proliferation were analyzed.
Results. Addition of $VEGF_{111}$ increased density of functional capillaries (P=0.01) 3 days after grafting. By double immunostaining of Ki-67 and von Willebrand factor, we demonstrated that proliferating endothelial cells were found in 83% of the $VEGF_{111}$ group compared with 33% in the control group (P=0.001). This angiostimulation was associated with a significant enhancement of hemoglobin content (P=0.03). Three weeks after transplantation, the number of primary follicles was significantly higher in $VEGF_{111}$ grafts (P=0.02).
Conclusion. $VEGF_{111}$ accelerates blood vessel recruitment and functional angiogenesis and improves the viability of ovarian cortex by limiting ischemia and ovarian cortex damage.

Keywords: Fertility preservation, Xenotransplantation, Angiogenesis, $VEGF_{111}$.

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Gonadotoxic effect of chemotherapy and radiotherapy on the ovarian function, resulting in premature ovarian failure, is well documented (*1, 2*) and the number of patients asking for fertility preservation dramatically increased. Fertility preservation has gained attention in the last few decades. The choice of an appropriate method of fertility preservation must be adapted for each patient according to the time available before cancer treatment, the age of the patient, the cancer type, and the treatment administered (*3*). Ovarian tissue freezing represents an alternative option when the delay to ovulation induction is not acceptable or contraindicated. More importantly, it is the unique accessible process for children

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or prepubertal women (*4*). Until now, 18 live births have occurred in women grafted with frozen ovarian tissue after cancer recovery (*5*).

Many animal investigations focused on the ovarian tissue survival improvement have been described. Transplantation of frozen/thawed ovarian tissue in animal models led to antral follicle development and live birth (*6–9*). However, the dramatic follicle loss after ovarian cortex transplantation may explain the limited success of this procedure. Indeed, fibrosis, apoptosis, and more specifically ischemia of ovarian graft monitored by electron paramagnetic resonance, affect follicular survival (*8, 10, 11*). Hypoxic period was identified during the first 5 days after transplantation followed by gradual oxygenation of the ovarian transplant over the next 5 days (*12*). Therefore, it appears crucial to minimize ischemic injury of the ovarian transplant by reducing the latency period before neovascularization establishment. In the ovary, primordial follicles do not possess their own capillary network but are dependent on proximal stromal vessels (*13*). Soon after the antrum formation, follicles acquire a vascular network in the theca interna and externa (*14*). Proliferation of the microvascular compartment in theca cell layer is essentially induced by vascular endothelial growth factor (VEGF) (*15, 16*), the main regulator of blood vessels growth during vasculogenesis and angiogenic sprouting. Indeed, a correlation between VEGF expression and the extent of angiogenesis and maturation of growing follicles has been described (*17*). VEGF-A protein is encoded by a gene containing eight exons. The *VEGF-A* gene can give rise to several isoforms ($VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$) resulting from a process of alternative splicing of the primary transcript (*18, 19*). The signal peptide and the domain interacting with VEGF receptor type 1 (VEGF-R1) and type 2 (VEGF-R2) are encoded by the four first exons, whereas the amino acid sequence encoded by exon 5 includes the site of cleavage by plasmin and metalloproteinases. Exon 6 encodes sequence that binds to heparin and cell surface while binding site to neuropilin-1 is encoded by exon 7 (*20*) or 8 (*21*). According to the presence or absence of exon 6 or 7, the most frequently detected isoforms can be diffusible ($VEGF_{121}$), only associated to cell surface and extracellular matrix ($VEGF_{189}$) or have intermediate characteristics ($VEGF_{165}$) (*22*). Recently, Mineur et al. (*23*) have identified a new VEGF-A isoform, $VEGF_{111}$, characterized by the lack of exons 5 to 7. This isoform is therefore highly diffusible and resistant to proteolysis. Consequently, this new VEGF-A isoform is documented to have a strong angiogenic activity in comparison with the other VEGF isoforms.

In the present study, we describe a new xenotransplantation model of cryopreserved ovarian tissue that allows the efficient in situ exposure of cryopreserved tissue to $VEGF_{111}$, which could stimulate angiogenesis and increase follicular preservation.

RESULTS

Ovarian Transplant Vascularization

Functional blood vessels were evidenced in transplants resected at 3 days and 3 weeks after transplantation by dextran/fluorescein isothiocyanate (FITC) perfusion followed by immunohistochemical detection of FITC (Fig. 1A). At day 3 after transplantation, in the presence of $VEGF_{111}$, 58% of ovarian transplants showed functional blood vessels identified by dextran/FITC staining. In the absence of $VEGF_{111}$, only 38% of the transplanted tissues were perfused by functional vessels (Table 1). Functional blood vessel density was determined by a computer-assisted image analysis as described previously (*24*). After 3 days, the density (number per square millimeter) of dextran/FITC-positive blood vessels was found to be significantly higher in the $VEGF_{111}$ group compared with control group (P=0.01; Fig. 1B). Macroscopic aspect (Fig. 1C,D) and hemoglobin dosage (Fig. 1E) confirmed that $VEGF_{111}$ enhanced blood vessel recruitment. Indeed, hemoglobin content (milligram hemoglobin per milligram lyophilized transplant) was significantly increased in $VEGF_{111}$-treated transplants compared with untreated transplants (0.405±0.131 vs. 0.535±0.139; P=0.03; Fig. 1E). These results confirm the positive effect of $VEGF_{111}$ on graft tissue perfusion and vessel connection between sheep ovarian graft and mice leading to a functional vascular network.

After 3 weeks, the mean functional vessel density was found to be significantly higher compared with that observed at day 3 and no difference was noted between the $VEGF_{111}$ group and the control group (Fig. 1B).

Intraovarian newly formed murine vessels were identified by using species-specific anti-mouse CD31 antibody. At day 3, $CD31^+$ murine vessels were detected only in the peripheral sheep cortex of $VEGF_{111}$ exposed grafts, whereas they were absent from the control samples (Fig. 1F). At week 3, the murine vascular density tended to be higher in the $VEGF_{111}$-treated group compared with the control group (P=0.06; n=11; Fig. 1G). Altogether, these results indicate that $VEGF_{111}$ exerts an angiostimulation in murine host tissue.

Cell Proliferation (Ki-67)

Cell proliferation was quantified in whole tissue sections by a computer-assisted image program. At day 3, Ki-67–positive stromal and endothelial cells were localized at the periphery of the ovarian grafts in both experimental groups (Fig. 2A, B). The percentage of vessels containing proliferative endothelial cells was significantly higher in VEGF-exposed grafts (84%) than in control transplants (33%; P=0.001; Table 1). Double immunostaining of Ki-67 and von Willebrand factor (vWF) confirmed the colocalization of the two markers in endothelial cells of $VEGF_{111}$-treated group (Fig. 2C,D). After 3 weeks, the proliferative index of stromal and endothelial cells was minimal (<1%) and did not show any significant difference between the two experimental groups (data not shown).

Follicular Density

Analysis of hematoxylin-eosin (H&E)–stained slide of cryopreserved/thawed ovarian fragments and samples recovered 3 weeks after transplantation showed that the density of primordial and primary follicles was significantly higher in nongrafted frozen/thawed samples compared with grafted frozen/thawed samples. These results clearly demonstrated the deleterious effects of ovarian tissue transplantation on follicular loss. Comparison of transplanted ovarian tissue in the control (n=12) and $VEGF_{111}$ (n=11) conditions demonstrated that $VEGF_{111}$ reduced follicular loss, which was statistically significant for the primary follicles (P=0.02; Fig. 3).

DISCUSSION

In the present study, we set up a xenotransplantation model of cryopreserved ovarian tissue and evaluated the

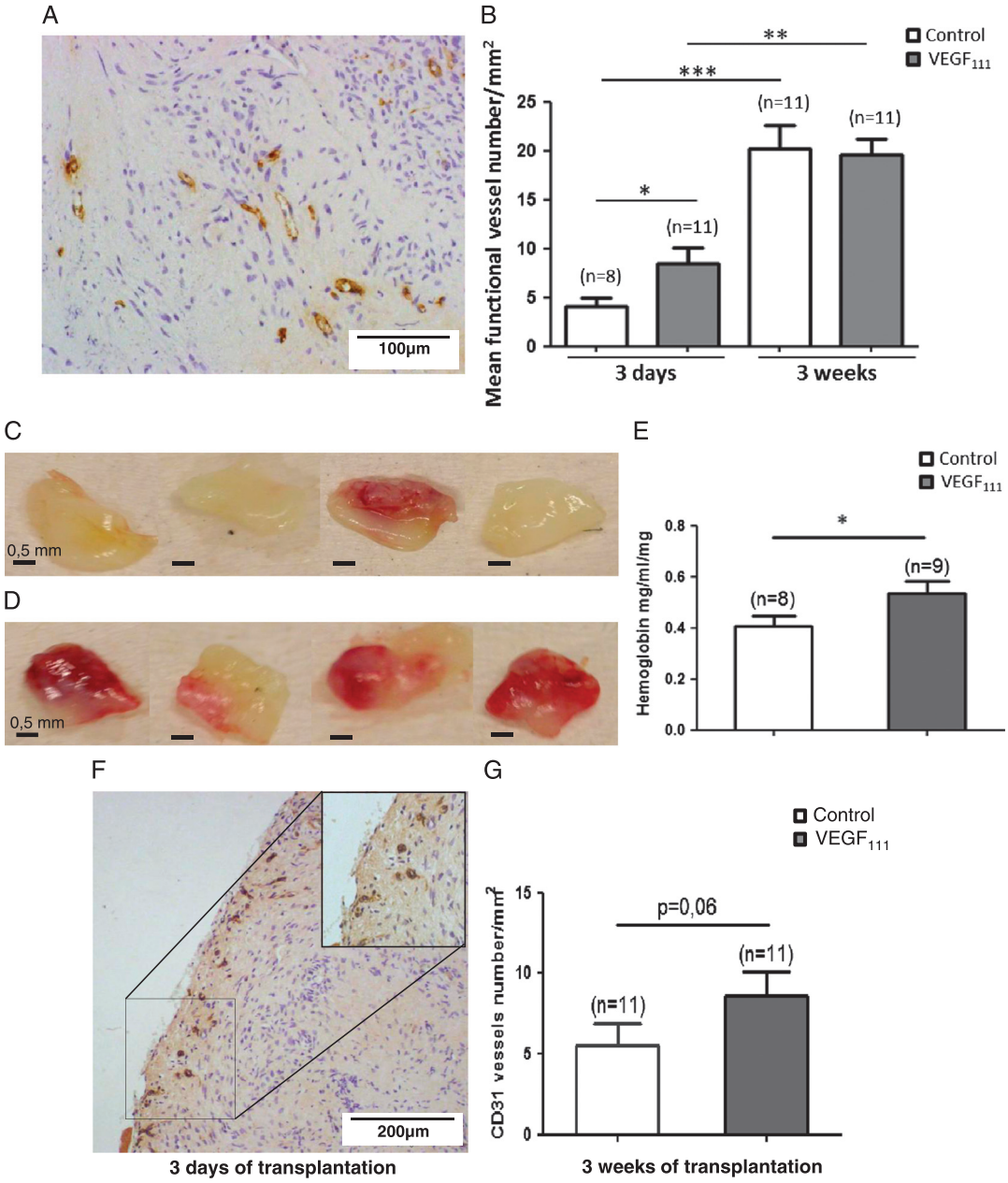

FIGURE 1. Vascularization analysis of transplants treated or not with $VEGF_{111}$. In the aim to analyze transplant neovascularization, mice were intravenously injected 3 min before sacrifice with dextran/FITC solution. A, illustration of functional blood vessels by dextran/FITC analysis on transplant section. B, dextran/FITC-positive blood vessel quantification 3 days and 3 weeks after transplantation. Representative image of untreated (control) group (C) and $VEGF_{111}$-treated ovarian grafts (D) recovered 3 days after transplantation. E, hemoglobin measurement evaluated by Drabkin's reaction in the ovarian cortex grafted for 3 days. F, Murine blood vessels stained by specific mouse CD31 in sheep ovaries grafts recovered 3 days after transplantation in presence of $VEGF_{111}$. G, quantification of murine blood vessel on sheep ovarian graft in control and $VEGF_{111}$ groups after 3 weeks of transplantation. n corresponds to the number of ovarian transplant analyzed per mouse. FITC, fluorescein isothiocyanate; $VEGF_{111}$, isoform 111 of vascular endothelial growth factor.

capacity of $VEGF_{111}$, a recently described VEGF-A isoform (*23*), to improve ovarian neoangiogenesis and follicular survival by reducing hypoxic period. First, we confirmed that transplantation of frozen/thawed cortex is associated with a significant loss of follicular density. Second, we demonstrated that $VEGF_{111}$ is able to induce vessels recruitment in ovarian transplant associated to an increase of the follicular survival.

Ischemia and fibrosis of ovarian tissue occurring after transplantation are the most important factors responsible for follicular loss, which affects hormonal environment and fertility restoration potential. Several options have been

TABLE 1. Percentage of ovarian grafts presenting functional blood vessels (dextran/FITC positive) and proliferated endothelial cells (Ki-67 positive) in control and $VEGF_{111}$ groups 3 days after transplantation

	Dextran/FITC, n (%)		Ki-67 endothelial cells, n (%)	
Groups	**Positive**	**Negative**	**Positive**	**Negative**
Control group (n=21)	8 (38)	13 (62)	7 (33)	14 (64)
$VEGF_{111}$ group (n=19)	11 (58)	8 (42)	16 (84)	3 (16)

FITC, fluorescein isothiocyanate; $VEGF_{111}$, isoform 111 of vascular endothelial growth factor.

investigated to reduce ischemic injuries during ovarian tissue transplantation such as administration of antioxidants and growth factors, either intraperitoneally or subcutaneously injected (*25*). The effect of hormones has also been evaluated on the follicular population, but results remain contradictory. No clinical answer has been addressed to improve ovarian graft vascularization, except when the graft was inserted into granulation tissue (*26*). Schnorr et al. (*27*) have previously tested the effect of daily $VEGF_{165}$ injection in monkey transplanted with ovarian cortex and did not observe any effect on graft vasculature. In contrast, recent reports demonstrated that host treatment with melatonin or graft incubation with hyaluronan, especially when combined with VEGF-A and vitamin E, improved graft survival and reduced apoptosis (*28, 29*). Israely et al. (*26*) reported that implantation of ovarian graft into an angiogenic granulation tissue, created during wound healing, improved graft vascularization and follicular survival. Inflammatory cells play a key role in this process. Platelet-derived cytokines recruit leukocytes and monocytes, which produce growth factors such as VEGF and basic fibroblast growth factor. Based on these results, this physiologic phenomenon was successfully applied for autotransplantation in women inducing new blood vessel formation by creating a peritoneal pocket or longitudinal opening of the ovary at the ovarian tissue transplantation sites 1 week before the transplantation (*30–32*). Similarly, it was shown that the systemic administration of sphingosine-1-phosphate to human tissue xenotransplantation increased neoangiogenesis and follicular survival (*33*). In spite of this encouraging result, sphingosine-1-phosphate is impractical in the clinic due to its dissolution in methanol or dimethylsulfoxide.

According to previous data demonstrating that transplanted ovarian cortex is hypoxic during the first 5 days of transplantation (*12*), we analyzed the impact of type I collagen (Col I) embedded with $VEGF_{111}$ on the vascular network and its possible effect on reducing this ischemic period. Indeed, $VEGF_{111}$ is characterized by high bioavailability and angiogenic activities due to its diffusible form and resistance to proteolysis (*23*) in comparison with $VEGF_{165}$. In addition, Col I gel used in our study was shown previously to support ovarian follicle growth (*34*). The delivery of $VEGF_{111}$ by the collagen matrix significantly increased the number of functional blood vessels in the grafted ovarian tissue already 3 days after transplantation. Indeed, functional blood vessels were

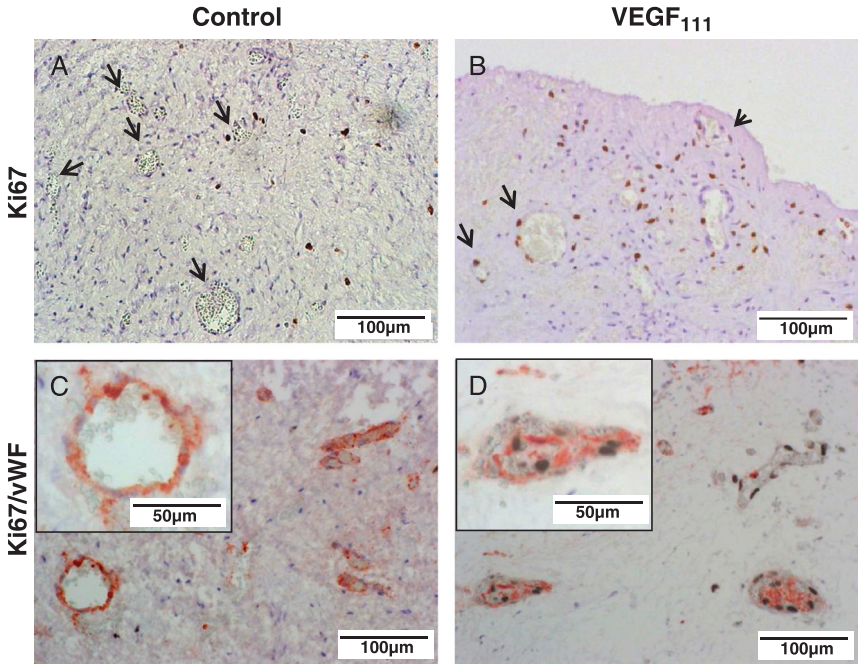

FIGURE 2. Endothelial cell proliferation analysis. Ki-67–negative endothelial cells were mainly detected in the control group (arrows; A) compared with Ki-67–positive endothelial cells in the $VEGF_{111}$ group (arrows; B). Double immunostaining of Ki-67/vWF in the control group (C) and $VEGF_{111}$-treated group (D). $VEGF_{111}$, isoform 111 of vascular endothelial growth factor; vWF, von Willebrand factor.

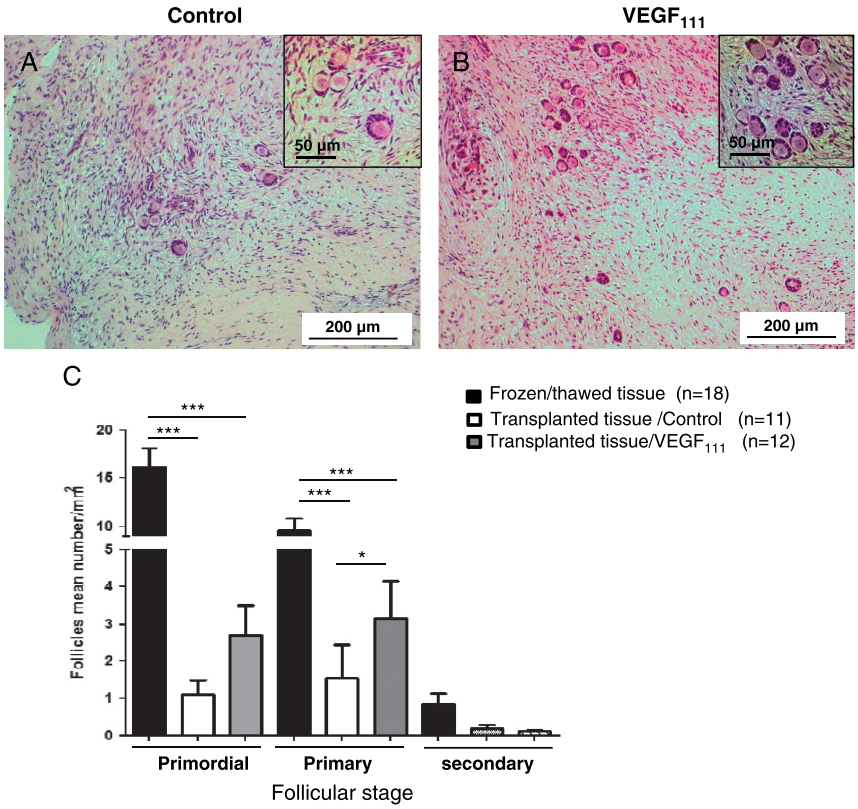

FIGURE 3. Follicle analysis of ovarian transplants. H&E section of ovarian transplants in control group (A) and $VEGF_{111}$ group (B). Primordial, primary, and secondary follicle quantification (mean number per square millimeter) in frozen/thawed control samples and in grafted fragments in the control and $VEGF_{111}$ groups (C). n corresponds to the number of ovarian transplant analyzed per mouse. H&E, hematoxylin-eosin; $VEGF_{111}$, isoform 111 of vascular endothelial growth factor.

identified in 58% of ovarian transplants treated with $VEGF_{111}$ and 33% in untreated transplants with a significant increase of vascular density in the $VEGF_{111}$ group. The existence of specific murine CD31 blood vessels observed in grafts of $VEGF_{111}$ group but not in control transplants supports the improvement of vascular anastomoses between sheep tissue and mice under the influence of $VEGF_{111}$. Such vascular anastomose is crucial for the success of ovarian transplantation leading to an early reduction of ischemic period, ovarian tissue damage, and follicular loss. The $VEGF_{111}$ isoform is known to be highly diffusible (*23*) and promotes early vessels recruitment. Therefore, 3 weeks after transplantation, the proangiogenic effect of $VEGF_{111}$ was not anymore observed. However, a significantly higher preservation of follicular reserve was observed in the $VEGF_{111}$ group at 3 weeks, probably related to the reduced hypoxic period.

Protective effects on follicles has been demonstrated by using vitamin E (*35*) and recent work has shown that treatment of mice during the first 5 days after transplantation with granulocyte colony-stimulating factor in conjunction with VEGF maintains primordial follicle number and prevents their loss in transplanted mouse ovaries (*36*). The vasculature network was not investigated in their study. Shikanov et al. (*37*) have also demonstrated that encapsulation of cryopreserved mice ovary in a fibrin matrix supplemented with mice $VEGF_{168}$ promotes ovarian graft survival.

In the present study, we provide evidence that $VEGF_{111}$ improved angiogenesis by stimulation of endothelial cell proliferation as demonstrated by double immunostaining of Ki-67 and vWF. Of interest is our finding that this effect on endothelial cells was associated with a significant increase of hemoglobin content in the graft, demonstrating the functionality of the neoformed vessels supported by dextran/FITC staining.

In conclusion, we demonstrate the usefulness of this ovarian encapsulation model to test the angiogenic capacity of several molecules directly on the transplanted tissue. Moreover, we provide evidence that ovarian cortex conditioned with $VEGF_{111}$ may accelerate blood vessel recruitment, a request to limit ischemia, ovarian cortex damage, and follicle loss and pave the way to human clinical application in the future.

MATERIALS AND METHODS

Collection of Ovarian Tissue

The use of ovarian sheep tissue and the xenograft model were approved by the ethics committee of the University of Liège. Ewe ovarian biopsies were

obtained from the Ovine Research Center (University of Namur [Facultés Universitaires Notre-Dame de la Paix]). After euthanasia, ovarian tissue was immediately transferred in Leibovitz L-15 medium (Lonza, Verviers, Belgium) supplemented with 10% normal sheep serum (Hormonology Laboratory, Marloie, Belgium) and kept at 4°C until processing.

Freezing and Thawing of Sheep Ovarian Tissue

Ovarian cortex from four sheep was prepared as described previously (*6*). Briefly, medulla was removed and cortex cut into strips of approximately 5×5×1 mm. For each ovary, one fragment was fixed in 4% formaldehyde solution for histologic analysis and the others were transferred in cryopreservative medium containing Leibovitz L-15 medium supplemented with 10% dimethylsulfoxide (Sigma-Aldrich, Bornem, Belgium), 10% normal sheep serum, and 0.1 M sucrose. After equilibration in cryopreservation medium for 30 min at 4°C, ovarian strips were placed in cryovial tubes (Simport, Montreal, Quebec, Canada) precooled at 4°C and subsequently cooled in a programmable freezer (CL-8800i System; CryoLogic, Mulgrave, Victoria, Australia) as described previously (*38*). For thawing, cryovials were removed from liquid nitrogen and held at room temperature for 2 min before immersion at 37°C, in a water bath, for 2 min. Ovarian slices were washed three times, for 5 min, in Leibovitz medium to remove cryoprotectant before further processing.

Ovarian Transplant Encapsulation

Ovarian cortex explants were encapsulated in three-dimensional collagen matrix as described previously for the aorta ring assay model (*39–42*). Briefly, agarose wells were performed by preparing 1.5% agarose VII solution (Sigma-Aldrich) and running of 30 mL in sterile 10 cm tissue culture Petri dish. After agarose polymerization, rings were punched by using first 17 mm and then 10 mm punchers. The center of ring was removed and agarose wells were placed in a new Petri dish. Col I was extracted from the tail tendons of rats by the methods described by Fusenig et al. (*43*). Collagen matrix was prepared by mixing 9 volumes of Col I (2.4 mg/mL), 1 volume of 10× minimal essential medium, and approximately 0.1 volume of 1 M NaOH to adjust the pH to 7.4. On a heating plate (37°C), a first layer of Col I was run. After polymerization, in the agar ring over the first Col I layer, frozen/thawed ovarian sheep explants, pierced with surgical thread, were placed and overlaid by a second layer of Col I (Fig. 4A).

$VEGF_{111}$ was produced and purified as described previously (*23*). Briefly, RNA purified from UV-irradiated HaCat cells was reverse transcribed using SuperScriptII and an oligo(dT) primer. The complete coding sequence of *$VEGF_{111}$* was amplified with Pwo DNA polymerase (Roche, Penzberg, Germany) and ligated in the *Not*I-*Nhe*I sites of a modified pCEP4 vector (Invitrogen, Carlsbad, CA). Plasmids were amplified in XL10-Gold ultracompetent bacteria (Stratagene, La Jolla, CA) and used to transfect HEK293. Stably transfected cells were selected in the presence of hygromycin and used for $VEGF_{111}$ production. Conditioned media containing an average of 500 μg $VEGF_{111}$ per liter were collected. Purification was performed by affinity chromatography using an immobilized specific anti-VEGF antibody. Purified $VEGF_{111}$ (3.5 ng/μL) was included in collagen of treated group with a final concentration of 350 ng per transplant.

Severe Combined Immunodeficient Mice Transplantation

Eight-week-old severe combined immunodeficient mice (Charles River, Saint-Germain-sur-l'Arbresle, France) were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Dorsal incision was performed, agar ring was removed, and one ovarian cortex encapsulated in collagen gel was stitched with 7-0 Prolene suture into the right mice ovary (Fig. 4B). To study functional vessels and possible anastomoses of sheep and mice vessels in the grafted ovary transplant, 200 μL dextran/FITC (2.5 mg/mL in phosphate-buffered saline; Sigma-Aldrich) was intravenously injected 3 min before sacrifice. Animals were euthanized by cervical dislocation after 3 days or 3 weeks of grafting. Ovarian cortex was recovered and fixed in 4% formaldehyde. Each graft embedded in paraffin was cut into serial sections of

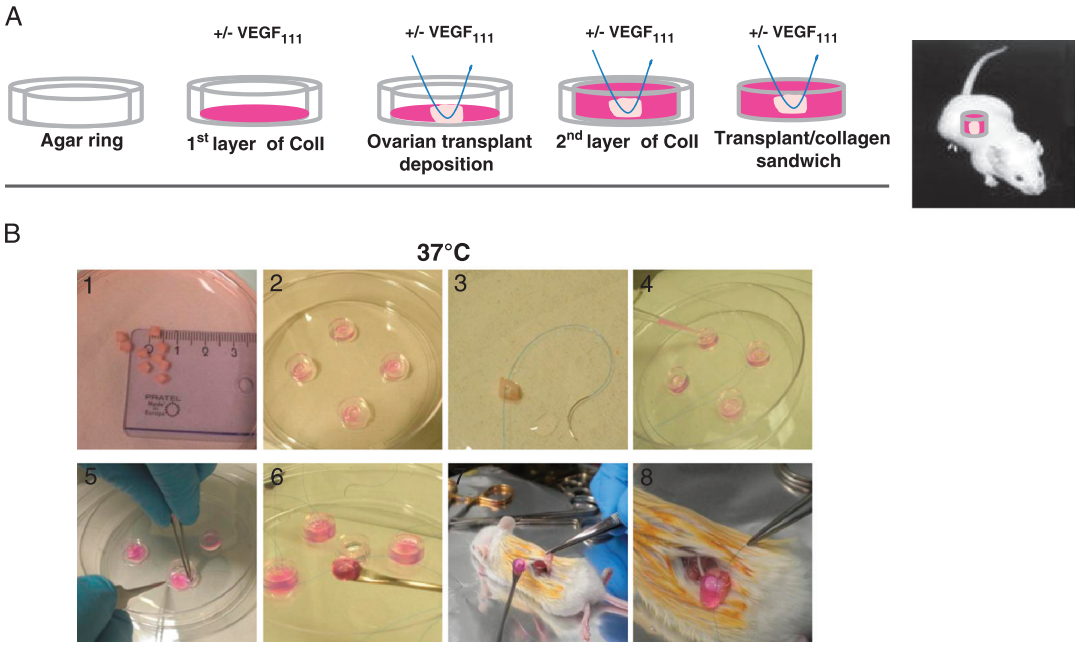

FIGURE 4. Steps of ovarian transplant encapsulation in collagen gels with or without $VEGF_{111}$ and xenograft into mice ovary as fully described in the Materials and Methods. On a heating plate (37°C), a first layer of Col I was run in agar ring. A, after polymerization, frozen/thawed ovarian sheep explants pierced with surgical thread were deposited and overlaid by a second layer of Col I gel. B, illustration of thawed sheep ovarian fragment followed by collagen encapsulation and grafting into severe combined immunodeficient mice ovary. Col I, type I collagen; $VEGF_{111}$, isoform 111 of vascular endothelial growth factor.

5 μm. Each tenth section was stained with H&E for histologic analysis (follicular classification and density) and the others were used for immunostaining.

Hemoglobin Measurement

Sheep ovarian cortex transplants were weighted, lyophilized, and homogenized for 3 hr in 100 μL of 0.1% saponin. Samples were spun at 10,000*g* for 15 min, and supernatants were collected. Hemoglobin content was evaluated using total hemoglobin kit (Sigma-Aldrich) Drabkin's reaction. Results are expressed as milligram hemoglobin per milliliter per milligram of fresh samples.

Immunohistochemistry

Immunohistochemical localization of vascular endothelial cells (CD31 and vWF), functional blood vessels (FITC/dextran), and proliferative cells (Ki-67) were performed with specific primary antibodies. CD31 antibody diluted 1:100 and incubated for 2 hr at room temperature (rat anti-mouse, ab56299; Abcam, Cambridge, MA) was used to identify murine blood vessels. Proliferated cells were stained by using monoclonal mouse anti-MIB1 antibody, diluted 1:100, and incubated for 1 hr at room temperature (M7240; Dako, Carpinteria, CA). A double immunostaining of proliferative cells and blood vessels was performed by using MIB1 antibody as described previously and a polyclonal rabbit anti-human vWF diluted 1:500 and incubated for 1 hr at room temperature (A0082; Dako). Paraffin sections were incubated for 30 min at room temperature with an anti-FITC TUNEL POD-labeled horseradish peroxidase (117724655001; Roche) to detect functional blood vessels in the ovarian transplant.

Specific binding was revealed using either Envision or an appropriate secondary antibody. Revelation was achieved with 3,3′-diaminobenzidine. For double immunostaining Ki-67/vWF, Ki-67 staining was revealed in gray with peroxidase substrate kit 3,3′-diaminobenzidine (SK-4100; Vector Laboratories, Burlingame, CA) and vWF staining in red with AEC system. For each specific immunohistochemistry, controls were performed by omitting the primary antibody or by incubating the sections with nonspecific IgG at the same concentration as the primary antibody.

Follicular Analyses

Whole scanned H&E sections were analyzed by Image J software. Follicles were classified and quantified in sheep cryopreserved/thawed ovarian graft before transplantation and after grafting in the control and $VEGF_{111}$-treated groups.

Section Analyses

Virtual images were acquired with the fully automated digital microscopy system dotSlide (BX51TF; Olympus, Aartselaar, Belgium) coupled with a Peltier-cooled high-resolution digital color camera (1376×1032 pixels; XC10; Olympus). Digital images of the whole tissue sections were digitized at high magnification (×100) producing virtual images in which pixel size is 1.510 μm. On digital images, vessels were drawn manually and transformed to obtain a binarized image in which pixels representing vessels have intensity equal to 1 and those corresponding to the background intensity have intensity equal to 0. On those binary images, the number of vessels was measured automatically. Results are expressed as unity of area of ovarian grafted tissue. Image analysis was conducted using the Matlab 7.9 software.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA). Mann-Whitney test was applied for comparison between two different groups. Statistical evaluation of the presence of blood vessels dextran/FITC-positive and Ki-67–positive endothelial cells between groups was performed using the chi-square test with Fisher's exact correction. Statistical significance was set at $P \leq 0.05$.

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REFERENCES

1. Maltaris T, Seufert R, Fischl F, et al. The effect of cancer treatment on female fertility and strategies for preserving fertility. *Eur J Obstet Gynecol Reprod Biol* 2007; 130: 148.
2. Oktay K, Sonmezer M. Chemotherapy and amenorrhea: risks and treatment options. *Curr Opin Obstet Gynecol* 2008; 20: 408.
3. Demeestere I, Moffa F, Peccatori F, et al. Multiple approaches for individualized fertility protective therapy in cancer patients. *Obstet Gynecol Int* 2012; 2012: 961232.
4. Maltaris T, Beckmann MW, Dittrich R. Review. Fertility preservation for young female cancer patients. *In Vivo* 2009; 23: 123.
5. Grynberg M, Poulain M, Sebag-Peyrelevade S, et al. Ovarian tissue and follicle transplantation as an option for fertility preservation. *Fertil Steril* 2012; 97: 1260.
6. Gosden RG, Boulton MI, Grant K, et al. Follicular development from ovarian xenografts in SCID mice. *J Reprod Fertil* 1994; 101: 619.
7. Gook DA, Osborn SM, Archer J, et al. Follicle development following cryopreservation of human ovarian tissue. *Eur J Obstet Gynecol Reprod Biol* 2004; 113: S60.
8. Schubert B, Canis M, Darcha C, et al. Follicular growth and estradiol follow-up after subcutaneous xenografting of fresh and cryopreserved human ovarian tissue. *Fertil Steril* 2008; 89: 1787.
9. Gook DA, McCully BA, Edgar DH, et al. Development of antral follicles in human cryopreserved ovarian tissue following xenografting. *Hum Reprod* 2001; 16: 417.
10. Nisolle M, Casanas-Roux F, Qu J, et al. Histologic and ultrastructural evaluation of fresh and frozen-thawed human ovarian xenografts in nude mice. *Fertil Steril* 2000; 74: 122.
11. Aubard Y, Piver P, Cogni Y, et al. Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. *Hum Reprod* 1999; 14: 2149.
12. Van Eyck AS, Jordan BF, Gallez B, et al. Electron paramagnetic resonance as a tool to evaluate human ovarian tissue reoxygenation after xenografting. *Fertil Steril* 2009; 92: 374.
13. Geva E, Jaffe RB. Role of vascular endothelial growth factor in ovarian physiology and pathology. *Fertil Steril* 2000; 74: 429.
14. Reisinger K, Baal N, McKinnon T, et al. The gonadotropins: tissue-specific angiogenic factors? *Mol Cell Endocrinol* 2007; 269: 65.
15. Yancopoulos GD, Davis S, Gale NW, et al. Vascular-specific growth factors and blood vessel formation. *Nature* 2000; 407: 242.
16. Ferrara N, Chen H, Davis-Smyth T, et al. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nat Med* 1998; 4: 336.
17. Otani N, Minami S, Yamoto M, et al. The vascular endothelial growth factor/fms-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. *J Clin Endocrinol Metab* 1999; 84: 3845.
18. Houck KA, Ferrara N, Winer J, et al. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 1991; 5: 1806.
19. Tischer E, Mitchell R, Hartman T, et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 1991; 266: 11947.
20. Soker S, Miao HQ, Nomi M, et al. VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. *J Cell Biochem* 2002; 85: 357.
21. Cebe-Suarez S, Zehnder-Fjallman A, Ballmer-Hofer K. The role of VEGF receptors in angiogenesis; complex partnerships. *Cell Mol Life Sci* 2006; 63: 601.
22. Ferrara N. Binding to the extracellular matrix and proteolytic processing: two key mechanisms regulating vascular endothelial growth factor action. *Mol Biol Cell* 2010; 21: 687.
23. Mineur P, Colige AC, Deroanne CF, et al. Newly identified biologically active and proteolysis-resistant VEGF-A isoform $VEGF_{111}$ is induced by genotoxic agents. *J Cell Biol* 2007; 179: 1261.
24. Balsat C, Blacher S, Signolle N, et al. Whole slide quantification of stromal lymphatic vessel distribution and peritumoral lymphatic vessel density in early invasive cervical cancer: a method description. *ISRN Obstet Gynecol* 2011; 2011: 354861.

25. Demeestere I, Simon P, Emiliani S, et al. Orthotopic and heterotopic ovarian tissue transplantation. *Hum Reprod Update* 2009; 15: 649.
26. Israely T, Nevo N, Harmelin A, et al. Reducing ischaemic damage in rodent ovarian xenografts transplanted into granulation tissue. *Hum Reprod* 2006; 21: 1368.
27. Schnorr J, Oehninger S, Toner J, et al. Functional studies of subcutaneous ovarian transplants in non-human primates: steroidogenesis, endometrial development, ovulation, menstrual patterns and gamete morphology. *Hum Reprod* 2002; 17: 612.
28. Abir R, Fisch B, Jessel S, et al. Improving posttransplantation survival of human ovarian tissue by treating the host and graft. *Fertil Steril* 2011; 95: 1205.
29. Friedman O, Orvieto R, Fisch B, et al. Possible improvements in human ovarian grafting by various host and graft treatments. *Hum Reprod* 2012; 27: 474.
30. Donnez J, Dolmans MM, Demylle D, et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet* 2004; 364: 1405.
31. Donnez J, Dolmans MM, Demylle D, et al. Restoration of ovarian function after orthotopic (intraovarian and periovarian) transplantation of cryopreserved ovarian tissue in a woman treated by bone marrow transplantation for sickle cell anaemia: case report. *Hum Reprod* 2006; 21: 183.
32. Demeestere I, Simon P, Buxant F, et al. Ovarian function and spontaneous pregnancy after combined heterotopic and orthotopic cryopreserved ovarian tissue transplantation in a patient previously treated with bone marrow transplantation: case report. *Hum Reprod* 2006; 21: 2010.
33. Soleimani R, Heytens E, Oktay K. Enhancement of neoangiogenesis and follicle survival by sphingosine-1-phosphate in human ovarian tissue xenotransplants. *PLoS One* 2011; 6: e19475.
34. Telfer E, Torrance C, Gosden RG. Morphological study of cultured preantral ovarian follicles of mice after transplantation under the kidney capsule. *J Reprod Fertil* 1990; 89: 565.
35. Nugent D, Newton H, Gallivan L, et al. Protective effect of vitamin E on ischaemia-reperfusion injury in ovarian grafts. *J Reprod Fertil* 1998; 114: 341.
36. Skaznik-Wikiel ME, Sharma RK, Selesniemi K, et al. Granulocyte colony-stimulating factor in conjunction with vascular endothelial growth factor maintains primordial follicle numbers in transplanted mouse ovaries. *Fertil Steril* 2011; 95: 1405.
37. Shikanov A, Zhang Z, Xu M, et al. Fibrin encapsulation and vascular endothelial growth factor delivery promotes ovarian graft survival in mice. *Tissue Eng Part A* 2011; 17: 3095.
38. Gosden RG, Baird DT, Wade JC, et al. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at −196 degrees C. *Hum Reprod* 1994; 9: 597.
39. Nicosia RF, Ottinetti A. Growth of microvessels in serum-free matrix culture of rat aorta. A quantitative assay of angiogenesis in vitro. *Lab Invest* 1990; 63: 115.
40. Masson VV, Devy L, Grignet-Debrus C, et al. Mouse aortic ring assay: a new approach of the molecular genetics of angiogenesis. *Biol Proced Online* 2002; 4: 24.
41. Berndt S, Perrier dHS, Blacher S, et al. Angiogenic activity of human chorionic gonadotropin through LH receptor activation on endothelial and epithelial cells of the endometrium. *FASEB J* 2006; 20: 2630.
42. Berndt S, Blacher S, Perrier d'Hauterive S, et al. Chorionic gonadotropin stimulation of angiogenesis and pericyte recruitment. *J Clin Endocrinol Metab* 2009; 94: 4567.
43. Fusenig NE, Limat A, Stark HJ, et al. Modulation of the differentiated phenotype of keratinocytes of the hair follicle and from epidermis. *J Dermatol Sci* 1994; 7 Suppl: S142.