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Mixed origin of neovascularization of human endometrial grafts in immunodeficient mouse models

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BACKGROUND: *In vivo* mouse models have been developed to study the physiology of normal and pathologic endometrium. Although angiogenesis is known to play an important role in endometrial physiology and pathology, the origin of neovasculature in xenografts remains controversial. The aim of this study was to assess the origin of the neovasculature of endometrial grafts in different mouse models.

METHODS: Human proliferative endometrium (n = 19 women) was grafted s.c. in two immunodeficient mouse strains: nude (n = 8) and severely compromised immunodeficient (SCID; n = 20). Mice were also treated with estradiol, progesterone or levonorgestrel. Fluorescence *in-situ* hybridization using a centromeric human chromosome X probe, immunohistochemistry (von Willebrand factor and collagen IV) and lectin perfusion were performed to identify the origin of the vessels.

RESULTS: More than 90% of vessels within xenografts were of human origin 4 weeks after implantation. Some vessels (9.67 ± 2.01%) were successively stained by human or mouse specific markers, suggesting the presence of chimeric vessels exhibiting a succession of human and murine portions. No difference in staining was observed between the two strains of mouse or different hormone treatments. Furthermore, erythrocytes were found inside human vessels, confirming their functionality.

CONCLUSION: This article shows that human endometrial grafts retain their own vessels, which connect to the murine vasculature coming from the host tissue and become functional.

Keywords: endometrium ; chimeric vessels ; fluorescence *in-situ* hybridization ; lectin ; angiogenesis

Introduction

Angiogenesis is the process by which new microvessels develop from existing blood vessels. Angiogenesis occurs by four different mechanisms that are sprouting, intussusception, vessel elongation and incorporation of circulating endothelial progenitor cells (EPCs) into growing vessels (Asahara *et al.,* 1997; Risau, 1997).

In the healthy human adult, angiogenesis is rare except in the female reproductive system where angiogenesis occurs in the ovarian follicle, corpus luteum and uterine endometrium (Gordon *et al.,* 1995; Fraser and Lunn, 2000; Jaffe, 2000). These tissues undergo cyclic changes to provide the nutrients and hormone precursors essential to the establishment and maintenance of pregnancy. In the endometrium, angiogenesis plays a key role in shedding and regrowth during the menstrual cycle (Abulafia and Sherer, 1999; Gargett and Rogers, 2001; Smith, 2001; Girling and Rogers, 2005). Abnormal angiogenesis may contribute to excessive bleeding in women with heavy menstruation, endometriosis, breakthrough bleeding and infertility (Donnez *et al.,* 1998; Krikun *et al.,* 2004; Mints *et al.,* 2005, 2007; Becker and DAmato, 2007; Saito *et al.,* 2007; Stephanie *et al.,* 2007). Angiogenesis is a crucial condition for the development and maintenance of endometriotic lesions: this role of angiogenesis has been recently demonstrated by Nap *et al.* (2004) using anti-angiogenic therapy in endometriosis induced in a mouse model.

A large variety of *in vivo* models using rodents have been developed to study the physiology of normal and pathologic endometrium. These models use the transplantation of autologous or heterologous endometrial

cells/tissues or endometriotic tissues (Story and Kennedy, 2004; Matsuura-Sawada *et al.,* 2005; Grummer, 2006; Masuda *et al.,* 2007). Immunocompromised animals do not possess the ability to reject foreign tissue and therefore appear as ideal candidates for such studies. According to the strain of mouse used to implant human endometrium s.c, the graft exhibits different morphological features. In nude mice, various degrees of fibrosis develop around the endometrial graft. On the contrary, human endometrial grafts in severely compromised immunodeficient (SCID) mice retain their normal histological features with no fibrotic reaction (Alvarez Gonzalez *et al.,* 2008). The main difference between the two strains of mice is that nude mice lack a thymus and cannot regenerate mature T lymphocytes whereas SCID mice lack both T and B lymphocytes. Differences in the fibrotic reaction between the two types of immunodeficient mice could modify angiogenesis in and around the endometrial graft, justifying investigation of graft vascularization in both strains.

In a previous study, we evaluated the effects of levonorgestrel (LNG) on human endometrium implanted in SCID mice (Alvarez Gonzalez *et al.,* 2008). LNG is considered as an efficient contraceptive delivered locally by an intrauterine device. LNG is known to alter the morphology and function of endometrium (Critchley *et al.,* 1998) as well as the endometrial vasculature by increasing blood vessel number, size and density and decreasing the pericyte coverage (Stephanie *et al.,* 2007), altogether contributing to vascular fragility and breakthrough bleeding (Hickey *et al.,* 2000; Rogers *et al.,* 2000; Jondet *et al.,* 2005). We demonstrated that human endometrial transplants reproduced similar histological characteristics and hormonal response when compared with eutopic human endometrium (Alvarez Gonzalez *et al.,* 2008).

Although angiogenesis is known to play an important role in endometrial physiology and pathology, the origin of neovasculature in xenografts remains controversial (Grummer *et al.,* 2001; Bruner-Tran *et al.,* 2002; Eggermont *et al.,* 2005; Masuda *et al.,* 2007).

In the present study, two immunodeficient mouse models were used to identify the origin of the neovasculature in grafts. Fluorescence *in-situ* hybridization (FISH), immunohistochemistry and lectin perfusion were performed to compare vessels in nude and SCID mice. As steroid hormones influence endometrial angiogenesis, progesterone and LNG were administered to the experimental animals.

Materials and Methods

Collection of human endometrium

The use of human tissue was approved by the Ethical Committee of the University of Liege, and an informed consent has been signed by all the women involved in the present study. Proliferative endometrium (5-9 cycle days) was obtained from 19 women of reproductive age (aged 31-38 years) without endometriosis undergoing surgery for benign purposes (uterine myoma). They had regular menstrual cycles and did not receive any hormonal treatment for at least 3 months before surgery.

Each endometrial biopsy was collected with a Cornier Pipelle suction curette (C.C.D. International, Paris, France), placed in sterile phosphate-buffered saline (PBS) solution, pH 7.3, and immediately transported to the laboratory. Part of the endometrial biopsy was fixed in 4% buffered formaldehyde, dehydrated and embedded in paraffin. Sections (5 µm-thick) were stained with haematoxylin-eosin for histological confirmation of the menstrual phase (Noyes *et al.,* 1950).

Animals

Experiments were approved by the Liege University Animal Ethical Committee.

Female SCID/SCID CB17 and Swiss nu/nu mice (8-10 weeks) were obtained from Charles River Laboratories (Brussels, Belgium) and housed under a barrier husbandry in a controlled pathogen free environment with a 12 h light/12 h dark cycle. All housing materials, food and water were autoclaved before use. Mice were fed *ad libitum* on laboratory chow and water.

Transplantation into mice and tissue processing

All surgical procedures were conducted in a sterile laminar flow hood under parenteral anaesthesia with an injection of a solution containing 10 mg/kg of Xylalin® and 80 mg/kg body weight of Ketamine 1000 Ceva® (both from Ceva Sante Animale, Libourne, France).

Eight nude mice were implanted s.c. in the neck region with a pellet of 17β-estradiol (E_2).

SCID mice were s.c. implanted either with E_2 alone (*n* = 8), with E_2+ progesterone (*n* = 4) or with E_2+ LNG (*n* = 8). E_2 and progesterone pellets were from Innovative Research of America, Sarasota, USA and contained, respectively, 1.72 and 25 mg of steroid, which were released at constant rate for 60 days. LNG was delivered by two poly-dimethylsiloxane implants inserted s.c. Each implant released 10 µg of LNG per day.

At least 2 days after insertion of the hormonal pellet, 10 fragments of fresh human proliferative endometrium (1-2 mm^3) per animal were grafted s.c. in the dorsal region of animals of both strains. Mice were sacrificed 3 or 4 weeks after endometrial transplantation. Depending on the size of the biopsy sample, 1 to 4 mice were grafted simultaneously.

The dorsal skin was removed and the adhering grafts were recovered and fixed in 4% buffered formalin and embedded in paraffin. Sections (5 µm-thick) were stained with haematoxylin-eosin for histological analysis or used for FISH and immunohistochemistry. Some grafts were fixed for 4 h at 4°C in 4% paraformaldehyde, cryopreserved in 30% sucrose/PBS overnight at 4°C and stored at -80°C until used for immunohistochemistry.

Fluorescence in-situ hybridization

Paraffin embedded sections were dewaxed in xylene and dehydrated in 100% ethanol. Sections were transferred for 20 min to 0.2 N HCl, washed in double distilled water (DDW) and in 2 x saline sodium citrate (2 x SSC, 0.3 M NaCl and 30 mM sodium citrate, pH 7.0, NV Invitrogen SA, Merelbeke, Belgium) for 3 min each and transferred to a pre-treatment solution (I N NaSCN, 71938, Fluka, Sigma-Aldrich NV/SA, Bornem, Belgium) at 80°C for 15 min. Sections were then washed in DDW for 1 min followed by 2 x 5 min in 2 x SSC Sections were incubated with Proteinase K (031 15879001, Roche SA, Brussels, Belgium; 0.25 mg/ml) at 37°C for 5 min, then washed in 2 x SSC for 2 x 5 min and fixed in 4% formaldehyde before being dehydrated through a 50 ml ethanol series: 70, 85 and 100% for 2 min each. Sections were denatured at 72°C for 5 min in 70% formamide/2 x SSC (pH 7) and then dehydrated in ethanol series as above.

The centromeric human chromosome X probe (CEP X Spectrum-Green, 32-132023, Vysis, Abbott SA/NV, Ottignies/Louvain-La-Neuve, Belgium) was denatured at 72°C for 5 min and then incubated at 37°C for 30-60 min.

Probe was applied to the section and then allowed to hybridize at 37°C overnight. Slides were washed in 2 x SSC/0.3% Tween 20 at 72°C for 2 min and then transferred to 2 x SSC at room temperature for 2 min. Ethanol series dehydration was performed as above and the slides were air dried in darkness. The slides were mounted and counterstained with Vectashield medium containing propidium iodide (H-1300, Vector Laboratories, Laboconsult SPRL, Brussels, Belgium).

Immunohistochemistry

Vascular studies were performed on 50 µm-thick cryosections. The human endothelial cells were stained using a rabbit polyclonal anti-human von Willebrand factor (vWF, A082, Dako, Glostrup, Denmark, dilution/200). All blood vessels were visualized using a vascular basement membrane antibody against human and mouse collagen IV (SIF 100, guinea pig polyclonal antibody produced in our laboratory, dilution 1/1000) (Van Cauwenberge *et al.,* 1983).

Human stromal cells were stained using mouse monoclonal anti-CD 10 antibody considered as a reliable and sensitive immunohistochemical marker for normal and neoplastic endometrial stroma (McCluggage *et al.,* 2001) (NCL-CDIO-270, Novocastra Laboratories Ltd, UK; dilution/50) on 5 µm-thick paraffin sections. To unmask antigens, slides were autoclaved for 11 min at 126°C and 1.4 bar in EDTA buffer. Endogenous peroxides were blocked by 3% H_2O_2 for 20 min and non-specific binding was prevented by incubation in PBS containing 10% bovine serum albumin (Fraction V, Acros Organics, NJ, USA) for 1h. Sections were incubated overnight at 4°C with the primary antibody.

Slides were then incubated 1h at room temperature with an appropriate secondary antibody: FITC-conjugated mouse anti-guinea pig (F5642, Sigma-Aldrich NV/SA, dilution 1/100), biotinylated goat anti-rabbit (E432, Dako, dilution 1/400) followed by incubation with Cy3-conjugated streptavidin (S6402, Sigma-Aldrich NV/SA, dilution 1: 1000) at room temperature for 1h, or biotinylated goat anti-mouse (E433, Dako, dilution/400) followed by incubation with streptavidin/horse-radish peroxidase complex (P0397, Dako, dilution 1:500) at

room temperature for 30 min. Staining was performed with 3-3' diaminobenzidine hydrochloride (K3468, Dako) for 3 min. Slides were finally mounted with Vectashield Dapi (H-1200, Vector Laboratories) or counterstained with haematoxylin and mounted with Eukitt medium for light microscopy observation. Negative controls were performed both by omission of the primary antibody and by its replacement by an appropriate isotype control (mouse immunoglobulin (Ig)GI, X0931, or rabbit Ig fraction, X0936, Dako).

Lectins

Between 3 and 4 weeks after grafting, a mixture of FITC-conjugated lyco-persicon esculentum tomato lectin (LEA-FITC, FL1171, Vector Laboratories) and tetramethylrhodamine B isothiocyanate (TRITC)-conjugated helix pomatia agglutinin (HPA-TRITC, L1261, Sigma-Aldrich NV/SA) at a final concentration of 1 mg/ml was injected into the tail vein of 12 anesthetized mice (three per strain and per treatment). LEA-FITC binds to the luminal surface of endothelial cells of humans and mice (Kawashima *et al.,* 1990; Zeng *et al.,* 1998; Murphy *et al.,* 1999; Pena *et al.,* 2000; Debbage *et al.,*2001) whereas HPA-TRITC binds to vascular endothelial cells in most mammalian species except human (Galili *et al.,* 988; Galili, 1989; Rydberg *et al.,* 1999; Debbage *et al.,* 2001). Mice were sacrificed 3 min later and lesions were immediately recovered and fixed for 4 h at 4°C in 4% paraformaldehyde. Grafts were then cryopreserved in 30% sucrose/PBS overnight at 4°C and stored at -80°C. Cryosections were cut at 50 µm thick, dried on Superfrost Plus slides (Fisher Scientific, Tournai, Belgium) for at least 18 h, rinsed with PBS and mounted in Vectashield Dapi or Propidium iodide (Vector Laboratories).

Analysis of endometrial vasculature

For each graft, 50 µm sections were double stained for vWF and SIF 100. RGB pictures were collected from these double-stained sections as well as from the lectin sections. All these were converted to eight bit gray scale images (fluorescence intensity range: 0-255) and analysed using ImageJ software (http://rsb.info.nih.gov/ij/). Total area stained by each antibody or by the lectins was binarized using an appropriate threshold for distinguishing pixels of the vasculature (score 1) from those of the background (score 0). The area measured for vWF (human cells) was expressed as a percentage of area measured for SIF 100 (human and mouse cells). The area measured for HPA-TRITC (mouse) was expressed as a percentage of the area measured for LEA-FITC (human and mouse). As no difference in endometrial vasculature staining was observed between the two strains of mouse or treatments, values were grouped and expressed as mean + SD (n = 12).

Results

Fluorescence in-situ hybridization

The origin of all components of the endometrial tissue developed after grafting was determined by FISH using a specific centromeric human chromosome X probe (Fig. 1). All glandular and stromal cells were FISH positive confirming their human origin (Fig. 1b). However, in the fibrotic area which developed around the endometrial tissue, the absence of FISH positive cells demonstrated that the fibroblasts were from murine origin.

By FISH technique, vessels can be visualized solely by the presence of autofluorescent erythrocytes. We observed FISH-positive cells bordering the lumen of all vessels identified in the endometrial stroma. In contrast, in the surrounding fibromuscular tissue, no such labelling was observed (Fig. 2). The FISH-positive cells were probably endothelial cells and the presence of erythrocytes suggested that this human-derived vasculature was functional.

***Figure I** Two consecutive sections through a subcutaneous human endometrial graft in a mouse host (3-4 weeks after grafting) were stained either by immunohistochemistry using a mouse monoclonal anti-human CD 10 antibody, which shows human stromal cells in brown (a) or by FISH using the centromeric human chromosome X probe (b).*
FISH labelling (green dots) is observed in endometrial glands (G) and stroma (S), but not in the fibrotic tissue (F) surrounding the endometrial lesions. The enlarged view in (b) corresponds to the area delineated in (a). Autofluorescent erythrocytes are easily visualized (large yellow bodies). Images are representative of n = 8 mice. Scale bar = 50 µm (a) and 40 µm (b).

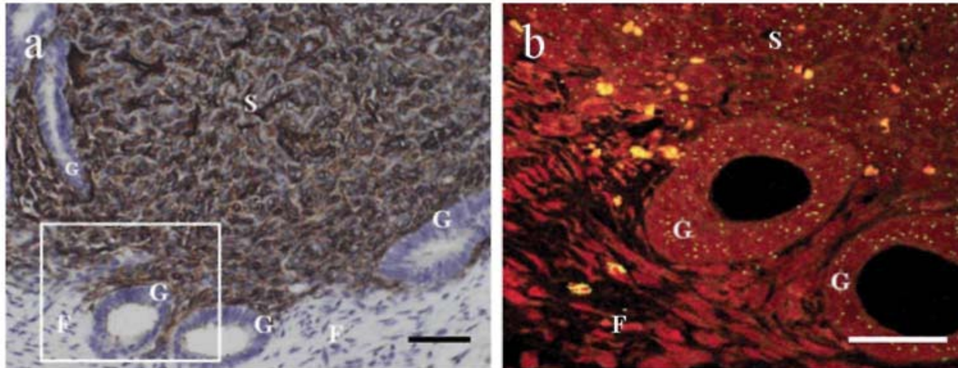
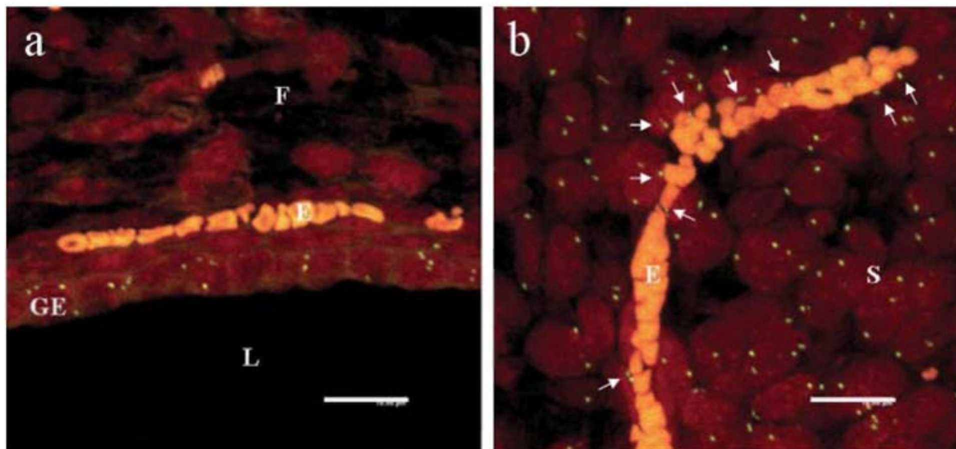


***Figure 2** FISH with centromeric human chromosome X probe in the human endometrial grafts in mice (green dots).*
No staining is observed in blood vessels located in the fibrotic tissue close to an endometrial gland (GE) of the graft (a). By contrast, positive staining is observed in the endometrial stroma and in vessels present in the stroma (arrows) (b). The vessels can be easily visualized by the presence of highly autofluorescent erythrocytes. Images are representative of n= 8 mice. L: lumen; GE: glandular epithelium; F: fibrosis; E: erythrocytes; S: stroma Scale bar = 16 µm.

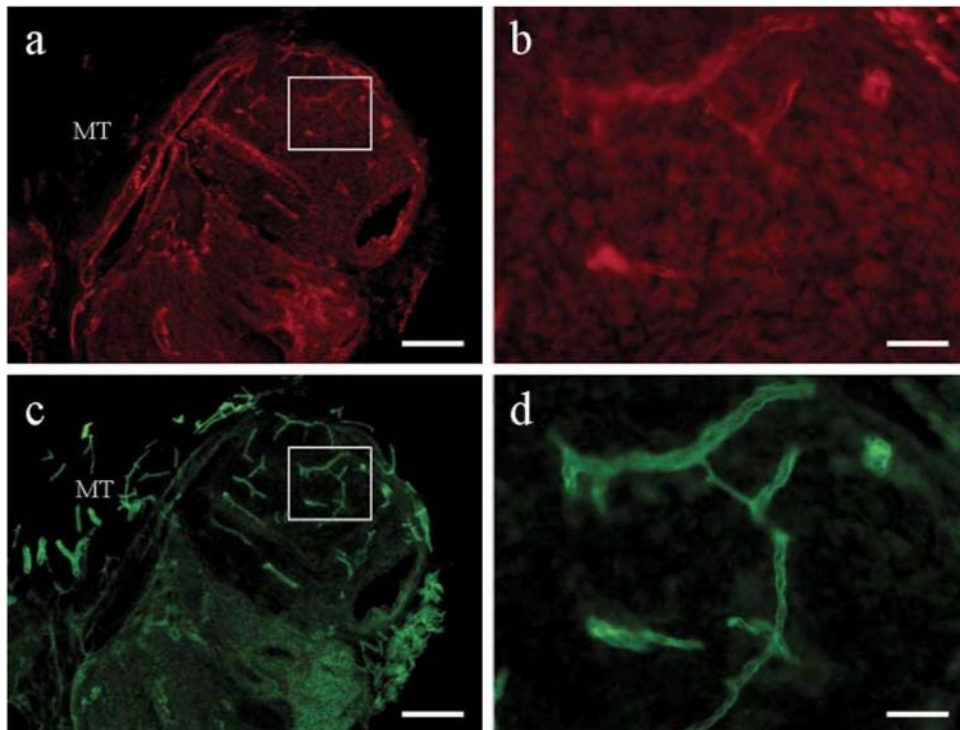


Immunohistochemistry

The vasculature was visualized in 50 µm sections by double immunos-taining of the endothelial cells (vWF) and the vascular basement membrane (SIF100) (Fig. 3). vWF was specific for human endothelial cells since no vessels were stained in the mouse tissue (MT) surrounding the lesion (Fig. 3a). The majority of vessels in the endometrial stromal tissue of the grafts were double stained (Fig. 3a and c). Closer observation of the sections highlighted the absence of vWF labelling in portions of vessels otherwise labelled with SIF 100 in a small

proportion (9.67 + 2.01%; *n* = 12) of vessels, suggesting their chimeric character, with portions of human and mouse origin (Fig. 3b and d).

Figure 3 *Double-immunohistochemical staining of a 50 µm-thick section through a subcutaneous human endometrial graft in mice with (a and b) rabbit polyclonal anti-human von Willebrand factor (vWF—red), labelling human endothelial cells, and (c and d) guinea-pig polyclonal anti-human and anti-mouse collagen IV (SIF 100—green), labelling the vascular basement membrane.*
Details of areas delineated in (a) and (c) are enlarged in (b) and (d), respectively. There are no vWF-positive cells in MT surrounding the graft (a). Within the endometrial stroma, the absence of vWF labelling in portions of vessels otherwise labelled with SIF 100 suggests their chimeric character (b and d). Images are representative of n = 12 mice. Scale bar = 100 µm in a and c, and 25 µm in b and d.



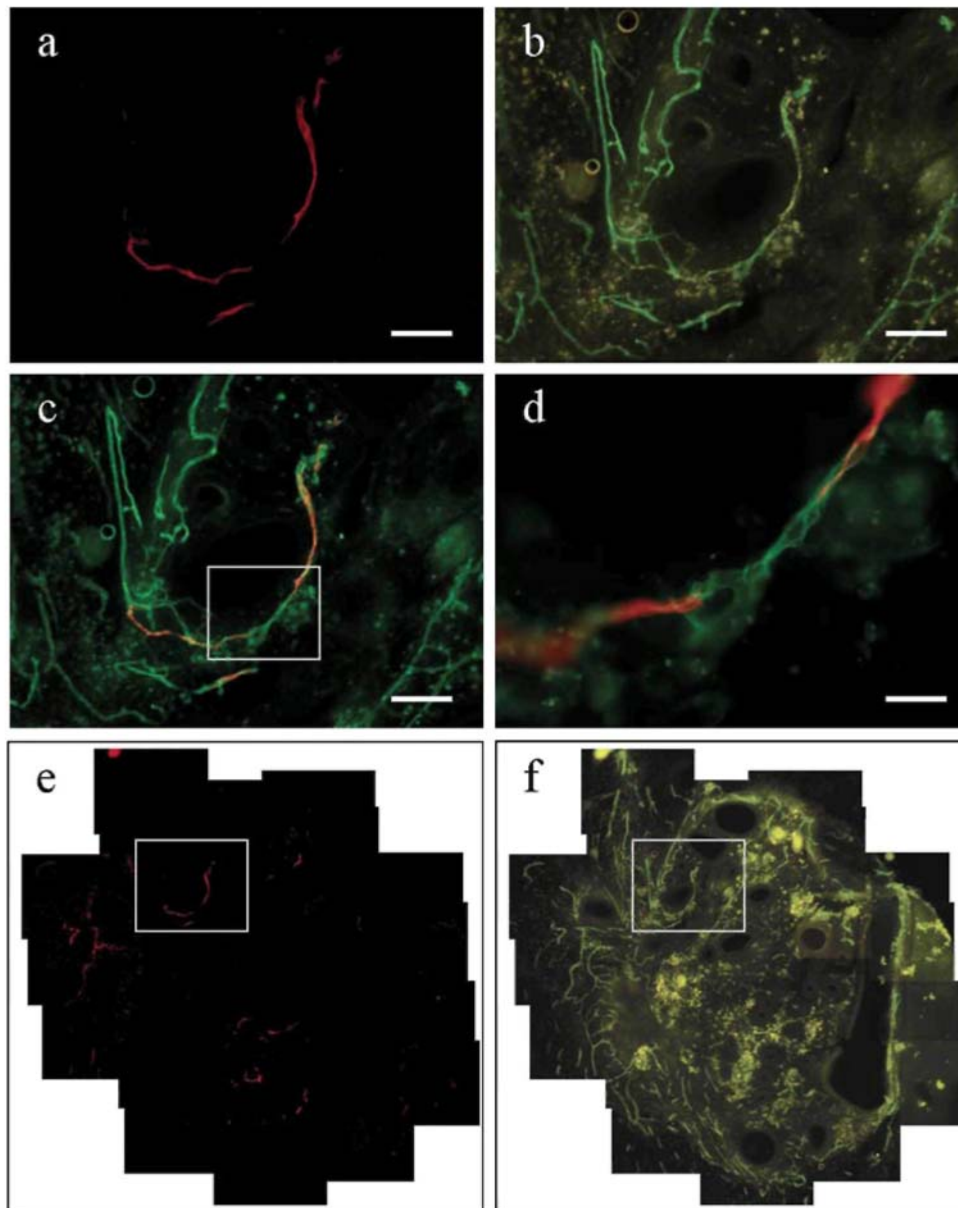
Lectins

To confirm the presence of chimeric vessels, we analysed 50 µm-thick sections obtained after simultaneous *in vivo* perfusion with LEA-FITC, staining human and mouse endothelial cells in green, and HPA-TRITC, staining only mouse endothelial cells in red (Fig. 4).

HPA-TRITC labelling was specifically observed in the MT surrounding the graft (data not shown). In the stroma, some vessels were stained by the two lectins. Closer observation revealed that these vessels exhibited successive portions that were either of murine (labelled by HPA-TRITC and LEA-FITC) or human (labelled only by LEA-FITC) origin (Fig. 4d). The success of the perfusion with the two lectins further confirmed that these vessels were functional.

No difference in vascularization was observed between mouse strain or hormonal treatments. In all groups, chimeric vessels were demonstrated in the endometrial stroma throughout the xenograft and not only at the edge next to the host MT (Fig. 4e). We never found chimeric vessels in the fibrotic tissue surrounding the lesions. The proportion of vessels from murine origin was estimated at 10.76 ± 1.93% (*n* = 12) thereby confirming the results obtained with the vWF/SIF 100 double labelling method (see above). The presence of erythrocytes in the vasculature enabled us to show that, as early as 3 weeks after the grafting procedure, human and murine blood vessels were connected and functional.

Figure 4 *Semithick section (50 μm) through subcutaneous human endometrial grafts in mouse simultaneously perfused with the lectin helix pomatia agglutinin tetramethylrhodamine B isothiocyanate (HPA-TR.ITC) (a), staining mouse endothelial cells, and with the FITC-conjugated lycoper-sicon esculentum tomato lectin (LEA-FITC) (b), staining both human and mouse endothelial cells.*
Single views in (a) and (b) are merged in (c) and (d), showing portions of vessels with different origins. Details of areas delineated in merged picture (c) are enlarged in (d). The whole section was reconstructed in (e) and (f) to show the limits of the graft either by HPA-TRJTC (e) or LEA-FITC (f). Small murine vessels (in red) are observed throughout the graft (e), i.e. at the edge and in the middle. The views in (a) and (b) correspond to the area delineated in (e) and (f). Images are representative of n = 12 mice. Scale bar = 100 μm in a, b, c, and 25 μm in d.



Discussion

This manuscript describes the presence of chimeric vessels (i.e. vessels exhibiting a succession of murine and human portions) throughout human endometrial expiants grafted in different mouse models.

Angiogenesis of human endometrial grafts is a crucial step for their survival and growth (Nap *et al.,* 2004). In

mouse models used to study endometrial pathologies, human endometrium is most often transplanted into either nude or SCID mice. The new vessels could then arise from human or mouse pre-existing vessels. Several authors evaluated angiogenesis in endometrial tissues transplanted in mice and showed that endometrial lesions derive their blood supply from the surrounding vascular network (Grümmer *et al.,* 2001; Bruner-Tran *et al.,* 2002). Nisolle *et al.,* (2000) evaluated angiogenesis with immunostaining using polyclonal anti-vascular endothelial growth factor (VEGF) antibody and observed the presence of some capillaries between the graft and the mouse peritoneal wall with VEGF-positive endothelial cells. However, these authors did not propose the presence of chimeric vessels. Grümmer *et al.,* stained vessels of human origin with anti-vWF antibody and endothelia of mouse vessels with a rat anti-mouse pan-endothelial cell antibody. They observed only vessels from mouse origin in endometrial lesions (Grummer *et al*., 2001). By staining endothelial cells with antibodies raised against human or mouse CD-31, Masuda et *al.* (Masuda *et al*., 2007) demonstrated human-mouse chimeric vessels in reconstructed endometrium following transplantation of dispersed endometrial cells beneath kidney capsule of ovariectomized NOD/SCID/γ_c^{null} (NOG) mice. Masuda *et al.* suggested that the lack of vessels of human endometrial graft origin in SCID and nude mice in previous studies (Grümmer *et al*., 2001; Bruner-Tran *et al.,* 2002; Hull *et al.,* 2003; Eggermont *et al.,* 2005) could be explained by the presence of functioning natural killer (NK) cells which would attack various immature precursor cells showing low levels of major histocompatibility complex expression. The lack of functional NK cells in NOG mouse would then allow the neovascularization of human origin to take place. Our experiments clearly demonstrated the presence of functional chimeric human-mouse vessels with circulating erythrocytes in nude mice as well as in SCID mice, and no major influence of hormonal treatment. Our results allow us to suggest that neovascularization of grafts does not seem to be dependent on the absence of NK cells.

Eggermont *et al.* (2005) demonstrated that revascularization of human endometrial grafts occurred between 5 and 8 days after implantation into nude mice and was characterized by the disappearance of native human graft vessels, coinciding with the invasion of the interface and then the endometrial stroma by murine vessels. In our study, by using semithick sections, grafts were fully evaluated, and endometrial stroma was clearly distinguished from the fibrotic tissue. We demonstrated the presence of chimeric human-mouse vessels throughout the grafts, mouse vessels being observed in the MT surrounding the grafts. We never observed chimeric vessels in fibrotic tissue around the grafts.

Lectins were described as suitable tools for intravital labelling of the vascular system (Debbage *et al.,* 2001). We simultaneously perfused mice with two specific lectins which recognized different ligands in the vascular endothelial glycocalyx (α-galactose and poly-*N*-acetyl-lactosamine). By using this technique, we labelled all the vessels and therefore followed them in the graft and determined their human and/or mouse origin. At the magnification used in Eggermont's study, the exact limit between the endometrial graft stroma and the surrounding mouse fibrotic tissue was difficult to assess. Therefore, their results could be explained by a progressive time-dependent increase of fibrosis in their endometriotic mouse model which is also observed in our nude mice (data not shown).

Very recently, Mints *et al.* (2008) showed that bone marrow-derived endothelial progenitors contribute to the formation of new blood vessels in the endometrium. They suggested that endometrial angiogenesis develops not only from local endothelial cells, but also from EPCs derived from bone marrow. The presence of mouse vessels connected with human vessels in our observations could be explained by the recruitment of mouse EPCs for the neovascularization of the endometrial lesions. Further studies are needed to assess this hypothesis.

In conclusion, by using several markers we observed that human endometrial expiants grafted into two different mouse models retained the vessels of human origin. These vessels connected to those of murine origin and became functional, as assessed by the presence of erythrocytes in the lumen and by the success of the simultaneous perfusion of the two lectins.

Author's Role

M.-L.A.G.: major contribution to conception and design; acquisition, analysis and treatment of data; drafting article; final approval of the version to be published. F.F.: substantial contribution to conception and design; revising critically the article; final approval of the version to be published. C.G.: contribution to the design of the model; revising of the manuscript. E.M.: contribution to the conception of the model; revising of the manuscript. J.-M.F.: substantial contribution to conception and design; revising critically the article; final approval of the version to be published. M.N.: substantial contribution to conception and design; revising critically the article; final approval of the version to be published. A.B.: drafting and revising critically the article; final approval of the version to be published.

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