The effects of ergot and non-ergot-derived dopamine agonists in an experimental mouse model of endometriosis

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Abstract

Implantation of a retrogradely shed endometrium during menstruation requires an adequate blood supply, which allows the growth of endometriotic lesions. This suggests that the development of endometriosis can be impaired by inhibiting angiogenesis. The growth of endometriotic foci is impaired by commercial oncological antiangiogenic drugs used to block vascular endothelial growth factor (VEGF) signaling. The dopamine agonist cabergoline (Cb2) inhibits the growth of established endometriosis lesions by exerting antiangiogenic effects through VEGFR2 inactivation. However, the use of ergot-derived Cb2 is associated with an increased incidence of cardiac valve regurgitation. To evaluate the potential usage of non-ergot-derived dopamine agonists for the treatment of human endometriosis, we compared the efficacy of quinagolide with that of Cb2 in preventing angiogenesis and vascularization in a heterologous mouse model of endometriosis. Nude mice whose peritoneum had been implanted with eutopic human endometrial fragments were treated with vehicle, 50 µg/kg per day oral Cb2, or 50 or 200 µg/kg per day quinagolide during a 14-day period. At the end of the treatment period, the implants were excised in order to assess lesion size, cell proliferation, degree of vascularization, and angiogenic gene expression. Neoangiogenesis was inhibited and the size of active endometriotic lesions, cellular proliferation index, and angiogenic gene expression were significantly reduced by both dopamine agonists when compared with the placebo. Given that Cb2 and quinagolide were equally effective in inhibiting angiogenesis and reducing lesion size, these experiments provide the rationale for pilot studies to explore the use of non-ergot-derived dopamine agonists for the treatment of endometriosis in humans.

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Introduction

Endometriosis is defined as the presence of endometrial tissue, glands, and stroma at sites other than the uterus. As in the case of the eutopic endometrium, the growth of ectopic lesions is hormonally regulated, causing periodic bleeding and inflammation associated with pelvic pain and infertility (Galle 1989).

Numerous peritoneal blood vessels surround active endometriotic lesions, and the implant itself is also endowed with a rich vascularized area; this can be observed under histological examination (Nisolle *et al.* 1993, Nap *et al.* 2004) and confirms the essential role that angiogenesis plays in the development and continuation of endometriotic lesions (Shifren *et al.* 1996, Donnez *et al.* 1998, Becker *et al.* 2005). In this regard, it has been observed that human endometrium engrafted in immunocompromised mice promotes the growth of neovessels from the surrounding host vascular

network in order to provide the endometriotic lesions with an adequate blood supply. Vascular endothelial growth factor (VEGF) acts through its VEGFR2 to play a pivotal role in the control of angiogenesis (McLaren *et al.* 1996) and so several antiangiogenic agents that target this pathway have been successfully used to disrupt the vascular supply of heterologous implants in immunodeficient animal models of endometriosis (Nap *et al.* 2004). However, the commercial anti-VEGF/VEGFR2 used in such experiments can induce severe side effects (Eremina *et al.* 2008), which rules out its use in the treatment of endometriosis in young and otherwise healthy women. Moreover, such compounds can also affect other important physiologic processes, including early pregnancy, by blocking implantation-related ovarian (Wulff *et al.* 2001, Zimmermann *et al.* 2001a, 2001b, 2003, Pauli *et al.* 2005) and uterine angiogenesis (Rockwell *et al.* 2002, Heryanto *et al.* 2003).

We hypothesized that dopamine receptor 2 (DRD2) agonists (DRD2-A) could be an alternative to commercial antiangiogenic agents. In animal models, DRD2-A inhibits pathologic angiogenesis in tumors (Basu *et al.* 2004) by inactivating VEGFR2 signaling (Basu *et al.* 2001). However, this type of medication has an acceptable safety profile and does not interfere with the normal establishment and progression of pregnancy (Robert *et al.* 1996, Ricci *et al.* 2002). We have previously used a well-established experimental endometriosis model to demonstrate that the DRD2-A cabergoline (Cb2) prevents the formation of typical endometriosis lesions by inhibiting angiogenesis (Novella-Maestre *et al.* 2009). These results encouraged us to perform a pilot study to evaluate the efficacy of Cb2 in the treatment of peritoneal endometriosis in humans.

An increased incidence of cardiac valve regurgitation has recently been associated with the use of ergot-derived DRD2-A such as Cb2 and pergolide in human subjects (Schade *et al.* 2007, Zanettini *et al.* 2007). These results have promoted the search for non-ergot-derived DRD2-A drugs with a more benign profile for the treatment of endometriosis.

In order to evaluate the potential benefits of non-ergot-derived DRD2-A for the treatment of human endometriosis, we assayed in a heterologous mouse model of endometriosis the efficacy of the DRD2-A quinagolide at 50 and 200 µg/kg doses in preventing angiogenesis and vascularization when compared with placebo or the ergot-derived Cb2 at 50 µg/kg as a comparator. We also evaluated in these four groups of animals whether or not the VEGF/VEGFR2 pathway was altered during the treatment.

Results

Evaluation of the endometriosis model

No toxic response to the treatment or signs of stress as a consequence of the handling and surgical procedures were observed. Similarly, no changes in behavior or significant variation in weight were detected among the different groups.

Of the four endometrial implants introduced per animal, 3.75 ± 0.25 were recovered per mouse, but

histological analysis revealed the histological hallmarks of endometriosis (glands and stroma) in only 3.5 ± 0.28 lesions per animal.

Histological analysis of lesions after Cb2 and quinagolide treatment

The lesion recovery rate was similar in all the groups studied, and no significant differences were detected among the groups with respect to the number of gland profiles per field in different implants or in the glandular/stromal area ratio. Both the percentage of active lesions and the lesion size decreased in both DRD2-A-treated groups when compared with controls (Table 1).

Immunohistochemical quantification of proliferation, vascular density, maturity, VEGFR2, and Drd2 expression

DRD2-A administration reduced proliferation of endometrial implants, which was evident in the significantly lower smaller area of nuclear Ki67 staining in the glands and stroma of the Cb2 ($P=0.020$) and the 50 µg/kg ($P=0.029$) and 200 µg/kg ($P=0.039$) quinagolide groups with respect to controls (Fig. 1 graph). However, this effect was not associated with a decrease in vascular density in any of the DRD2-A-treated groups (Fig. 2 graph). We did, nevertheless, detect a significant decrease in the number of immature vessels remaining in the DRD2-A-administered groups when compared with controls (Fig. 3 graph). Although no decreases were observed in the area occupied by vessel structures, the activation of DRD2 significantly reduced the area stained by VEGFR2 (Fig. 4 graph). Immunohistochemistry against DRD2 displayed a punctate pattern of staining in single cells and clusters of cells randomly distributed throughout the stroma. Given the fact that most of the 3,3'-diaminobenzidine (DAB; dark brown) staining for DRD2 appeared in hemosiderin deposits (light brown), we suspect that most of the cells expressing this receptor in the endometriotic lesions were macrophages (Fig. 5). The area of tissue stained against DRD2 in the endometriotic lesion tended to be

Table 1 Morphometric analysis of endometriotic-like fragments recovered from nude mice peritoneum in control, cabergoline 50 µg/kg (Cab), quinagolide 50 µg/kg (Quin50), and quinagolide 200 µg/kg (Quin200) high-dose groups.

	Control	Cab	Quin50	Quin200	P
Recovery rate (%)	93.7 ± 6.2	87.5 ± 7.2	93.7 ± 6.2	93.7 ± 6.2	0.84
Active lesion (%)	93.7 ± 6.2	47.9 ± 16.7	54.16 ± 11.0	60.41 ± 11.9	<0.05
Lesion size (mm ²)	9.64 ± 0.48	7.86 ± 0.38	7.77 ± 0.37	6.03 ± 0.39	<0.05
Glands/stroma	0.24 ± 0.04	0.22 ± 0.07	0.19 ± 0.06	0.22 ± 0.02	0.97
Glands/implant	5.00 ± 0.97	5.86 ± 2.65	4.38 ± 1.60	4.33 ± 1.08	0.89

Recovery rate %, percentage of implanted endometrial fragments that were recovered after the treatments; active lesion %, percentage of lesions showing the simultaneous presence of glands and stroma. Lesion size was calculated by multiplying the width and the length of the lesions recovered (expressed in mm²). Glands/stroma, ratio of the areas occupied by glands and stroma in active lesions; glands/implant, mean number of glands × microscopic field (10×) analyzed in active lesions. Results are expressed as mean ± S.E.M.

larger in DRD2-A-treated animals and reached statistical significance in the 200 µg/kg per day quinagolide group (Fig. 5 graph).

Real-time fluorescence-based quantitative PCR

VEGF, *VEGFR2*, and *Drd2* gene expression was evaluated by RT-PCR in order to detect expression at the mRNA level. Of the three transcripts quantified, *VEGFR2* was the only one that exhibited significant statistical differences ($P < 0.05$) in all DRD2-A treated groups (decreased mRNA expression levels) when compared to controls. Surprisingly, *VEGF* mRNA levels were significantly lower in the quinagolide- but not in the Cb2-treated groups with respect to controls. We observed a marked tendency for *Drd2* mRNA to rise with the higher doses of quinagolide, but these increases were not statistically significant when compared with controls (Fig. 6).

Discussion

The main goal of this study was to evaluate whether a non-ergot-derived DRD2-A-like quinagolide is as potentially effective as the ergot-derived DRD2-A Cb2 in preventing endometriotic angiogenesis by blocking the VEGF/VEGFR2 system. Interestingly, we found that quinagolide was equally effective as Cb2 in decreasing lesion size and proliferation and in decreasing *VEGFR2* mRNA and *VEGF* mRNA levels.

We focused our attention on the VEGF/VEGFR2 signaling pathway, as accumulated evidence suggests that DRD2-A binding to the DRD2 expressed on endothelial cells can inactivate VEGFR2 in an autocrine fashion, thereby avoiding its mediated angiogenic actions (Basu *et al.* 2001). Our results provide the first solid evidence of an alternative mechanism of action of the DRD2-A quinagolide by which it decreases angiogenesis. Indeed, we herein report that quinagolide decreases the expression of *VEGF* mRNA levels. Based on the punctate expression pattern of DRD2 staining observed in human endometrial implants and our unpublished results using human endometrial biopsies obtained throughout the whole menstrual cycle, we do not believe that this receptor is consistently expressed by the glandular epithelium, stroma, or endometrial vessels. On the contrary, we believe that DRD2 is expressed by cells of an immune origin – perhaps macrophages – and that these cells are probably responsible for inducing, in an autocrine and/or paracrine fashion, a decreased expression of VEGF when activated by DRD2-A. In this context, previous work by our group (Ferrero *et al.* 2010) and other authors (Cristina *et al.* 2005) has shown that cell populations expressing DRD2 (granulosa or pituitary cells) inhibit the secretion of VEGF in either an autocrine or a paracrine fashion. In this regard, it is known that most immune cell subtypes express DRD2 (McKenna

A

B

Figure 1 Cell proliferation of implants was investigated using the Ki67 labeling area (A). Representative pictures of immunohistochemical detection of Ki67 (brown color) in endometriotic-like implants from DRD2-A-treated and control mice. Note how cell proliferation is decreased in endometriotic implants of all DRD2-A-treated mice versus controls. Human neuroblastoma was used as a positive control for Ki67. (B) Percentage of stained area represented by Ki67-positive cells per area of interest, * $P < 0.05$.

et al. 2002), and among them, macrophages are known to express large amounts of VEGF (Nap *et al.* 2004). Moreover, the ability of macrophages to reduce angiogenesis when activated by dopamine has recently been described (Chakroborty *et al.* 2008). Thus, in our experiments, it is likely that quinagolide decreased *VEGF* transcripts in implanted endometrial tissue by

Figure 2 (A) Representative pictures of vascularization in endometriotic-like implants in DRD2-A-treated and control mice. Vessels were immunohistochemically detected using the endothelial marker CD31 (brown color). (B) Microvascular density of endometriotic-like implants in DRD2-A-treated and control mice was determined through quantification of the area stained by CD31 antigen per area of interest.

inhibiting the expression of this factor in macrophages through the activation of DRD2.

We have also shown that quinagolide decreases the expression of not only VEGF but also *VEGFR2* mRNA levels and that this is associated with a reduction in the area of the heterologous implant immunostained against the VEGFR2 protein. In accordance with previously reported data, the pattern of VEGFR2 immunodistribution observed in our endometriosis-like tissue was also exhibited in non-vascular cells, such as those in the glandular epithelium and interstitial stroma (Meduri *et al.* 2000, Bourlev *et al.* 2006, Punyadeera *et al.* 2006, Machado *et al.* 2008, 2010). Given that DRD2 does not seem to be consistently expressed in VEGFR2-positive structures, we hypothesize that the reduction in VEGFR2 expression in cell types expressing this receptor was exerted by DRD2-A in a paracrine fashion.

The growth of newly formed blood vessels is of pivotal importance in the development of endometriosis

(Nap *et al.* 2004). Angiogenesis involves complex molecular interactions between endometrial tissue, peritoneal tissue, infiltrating leucocytes, and fibroblasts (Hull *et al.* 2008). In addition, the origin of neovasculation in xenografts remains unclear, which further complicates the analysis of these interactions. Some authors have previously stated that human endometrial tissues transplanted in mice derive their blood supply from the surrounding vascular network, with the original human vessel simultaneously disappearing as the mouse vessels invade the stroma (Grümmer 2001, Bruner-Tran *et al.* 2002, Hull *et al.* 2003, Eggermont *et al.* 2005). However, recent studies have revealed the presence of mouse, human, and functional chimeric human–mouse vessels throughout such grafts (Masuda *et al.* 2007,

Figure 3 Effects of dopamine agonists on neoangiogenesis. (A) Representative pictures of double immunofluorescence for CD31 (green) and alpha smooth muscle actin (ACTA2, also known as α -SMA; red) in endometriotic-like implant sections from controls and DRD2-A-treated mice. White arrows point to immature vessels showing staining for CD31 (green) but lacking staining for ACTA2 (red). Mature vessels show double staining for CD31 and ACTA2. (B) Percentage of newly formed vessels in each group, represented as immature vessels per field. * $P < 0.05$.

Figure 4 Effects of dopamine agonists on VEGFR2 expression. (A) Representative pictures of immunohistochemical detection of VEGFR2 (brown) in endometriotic-like implants from DRD2-A-treated and control mice. VEGFR2 staining was observed in stroma, glands, and blood vessels (black arrows). Detailed VEGFR2 staining in vessel structures in representative images from each group is shown in a small box. Note the decreased expression of VEGFR2 in DRD2-A-treated versus control groups. (B) Estimation of VEGFR2 expression through quantification of VEGFR2-stained area per area of interest in DRD2-treated and control groups. * $P < 0.05$.

Álvarez-González *et al.* 2009, Guo *et al.* 2011). Similarly, host-derived cell types, such as macrophages and myofibroblasts, have been shown to coexist with macrophages from human endometrium embedded in the stroma of immunocompromised mouse xenografts (Hull *et al.* 2008, Guo *et al.* 2011). Such studies reveal an interaction between host and graft at different levels, by which a combined structure capable of responding to different stimuli is created. In the context of this interaction, the objective of our study was to evaluate the angiogenic response as a holistic process in which

both human and mouse tissues are implicated. Thus, antibodies and primers were selected to determine both human and mouse contributions, respectively, thereby obtaining a wider perspective of this process.

As was the case in previous experiments performed in the same heterologous animal model of endometriosis and in which we assayed different doses of Cb2 (Novella-Maestre *et al.* 2009), we observed no reduction in the area occupied by vessels but did find a decrease in the number of newly formed vessels in response to DRD2-A. We speculate that this behavior is a result of mature 'major' vessels being less sensitive to the actions

Figure 5 Effects of dopamine agonists on DRD2 expression. (A) Representative pictures of immunohistochemical detection of DRD2 (brown) in endometriotic-like implants from DRD2-A-treated and control mice. DRD2-A showed a punctate immunohistochemical pattern probably associated with scattered macrophages going through the stroma. Note the increased expression of DRD2 in DRD2-A-treated versus controls groups. (B) Estimation of DRD2 expression through quantification of DRD2-stained area per area of interest in DRD2-treated and control groups. * $P < 0.05$.

Figure 6 Comparison of (A) *VEGF*, (B) *VEGFR2*, and (C) *Drd2* mRNA levels in quinagolide- and cabergoline-treated versus control animal groups, determined by QF-PCR. AFU, arbitrary fluorescence units. * $P < 0.05$.

of VEGF due to their pericyte (ACTA2+) coverage, while newly formed 'small' vessels, which are free of pericytes, are more sensitive to VEGF (Benjamin *et al.* 1998). As DRD2-A reduced *VEGF* mRNA levels, it is feasible that quinagolide and Cb2 inhibited the revascularization process, which took place in the engrafted endometrium. As the specific origin of newly proliferating cells is unknown, we used antibodies to detect both human and murine blood vessel markers in order to analyze the contribution of both species to the hypothetical anti-angiogenic effect of DRD2. We observed a slight shrinkage in the size of peritoneal lesions treated with DRD2-A. Thus, we cannot rule out that the reduction in the vascularized area induced by DRD2-A, by taking place in vanished/regressed tissue, even in mature

vessels, was masked. In fact, a significant decrease in the proliferation index induced by DRD2-A, as shown by decreased Ki67 staining, suggested that effects of Cb2 and quinagolide, which were inhibitory for angiogenesis, were ongoing in the xenograft. We speculate that by increasing the period of Cb2 and quinagolide treatment in the nude model, it may be possible to detect decreases in vascularization. However, due to the characteristics of the animal model used and the constraints of time in this study, the prolonged survival of implanted tissue was not possible.

Despite strong evidence to suggest that DRD2 activation modulates angiogenesis by directly interfering with VEGFR2 signaling (Basu *et al.* 2001), it is plausible that dopamine agonists also inhibit angiogenesis in ectopic implants through alternative mechanisms. In this regard, and given that prolactin (PRL) secretion has been shown to promote angiogenesis by signaling through specific blood vessel receptors (Reese *et al.* 2000), it is possible that the reduction of circulating PRL levels as a result of DRD2 also contributes to the inhibition of the angiogenic response induced by these drugs in endometrial implants. Although there are several reports of an association between endometriosis and hyperprolactinemia (Gregoriou *et al.* 1999, Cunha-Filho *et al.* 2002), the existence of a fundamental proangiogenic role for PRL in human endometrial ectopic tissue is yet to be confirmed.

We did not evaluate whether DRD2-A affected angiogenesis in the reproductive organs, but such an action is unlikely in light of previous results published by our group after using tenfold higher DRD2-A doses than those used in this study, which showed that luteal angiogenesis and function were not compromised (Gómez *et al.* 2006). Further evidence of the inexistence of the detrimental effects of DRD2-A for eutopic endometrial angiogenesis is provided by the fact that women receiving high doses of Cb2 become pregnant uneventfully (Verhelst *et al.* 1999). Given its different mechanism of action, DRD2-As are unlikely to interfere with the menstrual cycle and thus possesses an important advantage over GnRH agonists and aromatase inhibitors, which induce osteoporosis and hot flashes as a result of hypoestrogenic milieu (Gregoriou *et al.* 1997, Walch *et al.* 2009).

In conclusion, the successful inhibition of ectopic endometrial growth by both types of DRD2-A under study in a immunocompromised mouse model provides the rationale for a pilot study in humans. Our results suggest that quinagolide is as effective as Cb2 in inhibiting the growth of endometriotic lesions in endometriosis patients. Moreover, as quinagolide is not an ergot derivative, it offers an additional advantage over Cb2, as it does not produce the side effects that ergot derivatives exert on the serotonin (5-hydroxytryptamine (5-HT)) receptor subtype 5-HT2b. Unlike Cb2, quinagolide is unlikely to affect cardiac valve tissue and,

therefore, is not expected to induce proliferation of fibroblasts (Roth 2007) or regurgitation. In addition, quinagolide has a much shorter half-life (~ 17 h) than Cb2 (~ 63 – 69 h; Colao *et al.* 2002), thus minimizing exposure during organogenesis for women who wish to become pregnant while under treatment with DRD2-A. Inducing a hyperprolactinemic state in the event of DRD2-A proving ineffective in the treatment of women with endometriosis may be ethically questionable. To avoid such controversy, we propose assaying these drugs in women suffering simultaneously from endometriosis and hyperprolactinemia, as DRD2-A therapy is indicated for both these conditions.

Materials and Methods

This study was approved by our Institutional Review Board and the informed consent of subjects was obtained prior to endometrial biopsy collection. Similarly, all the procedures using animals were performed according to the European Directive 86/609/CEE and NIH Guidelines for the Care and Use of Laboratory Animals.

Experimental model of endometriosis

The model of endometriosis was prepared as described previously (Nap *et al.* 2004) with minor modifications. A total of 34 ovariectomized 5-week-old female mice (Hsd: athymic Nude-nu; Harlan Ibérica S.L, Barcelona, Spain) were individually housed in autoclaved cages (with bedding) in laminar flow filtered hoods. The animal room was maintained at 26 °C with a 12 h light:12 h darkness cycle, and mice were fed with autoclaved laboratory rodent chow and acidified water *ad libitum*. All handling was performed in laminar flow filtered hoods. A mixture of 75 mg/g ketamine (Ketolar, Parke–Davis, Barcelona, Spain) and 1 mg/g medetomidine (Domtor, Pfizer, Madrid, Spain) was injected i.p. to anesthetize mice before invasive procedures. Atipamezole (1 mg/g; Antisedan, SmithKline Beecham, Madrid, Spain) was administered i.p. after invasive procedures to reverse the effects of anesthesia. Sixty-day-release sterile capsules containing 18 mg 17 β -estradiol (Innovative Research of America, Sarasota, FL, USA) were placed s.c. in the neck of each animal. Four days later, fresh human endometrium fragments were acquired via biopsy during ovum retrieval from four oocyte donors with normal menstrual cycles and no history of endometriosis (18–34 years old). These fragments, after being minced into pieces of $\sim 3 \times 3$ mm, were attached to the wall of the peritoneal cavity of each mouse using *n*-butyl-ester cyanoacrylate adhesive (Vetbond; 3M Animal Care Products, St Paul, MN, USA). Human endometrial samples from individual donors were used in at least two animals from each experimental group; in this way, four implants were introduced per animal. Three weeks after establishment of lesions, the animals were divided into four groups: three experimental and one control. In the experimental groups, 50 μ g/kg per day of Cb2 (Dostinex, Pfizer Labs) or 50 or 200 μ g/kg per day of quinagolide (Norprolac, Ferring Pharmaceuticals) were administered by oral gavage for

14 days. The dose of Cb2 that we used has previously been shown to be effective in inhibiting angiogenesis in the same endometriosis mouse model (Novella-Maestre *et al.* 2009) and was used as a reference for selecting the quinagolide doses assayed. The 50 μ g/kg dose of quinagolide was chosen in order to evaluate the effects of this drug when administered at the same daily doses as Cb2. Given that Cb2 has a higher half-life than quinagolide (Colao *et al.* 2002), the 200 μ g/kg dose of quinagolide was selected in an attempt to compare the effects exerted by similar accumulative doses of both compounds by the end of the treatment. Animals in the control group were administered with vehicle solution. The animals were killed by cervical dislocation 24 h after the last administration of drug or vehicle, and the abdominal skin and peritoneum were opened to examine the visceral organs under a binocular microscope and thus evaluate the presence of endometriotic implants and vascularization. The size of all the lesions in the peritoneum of mice with possible endometriosis was estimated, as described below. One half of the animals ($n=17$) were used for microscopic histological, immunohistochemical, and subsequent morphometric quantitative studies, and the remaining half ($n=17$) underwent the same procedures to obtain tissue, which was frozen at -80 °C for subsequent molecular analysis of angiogenesis-related genes.

Macroscopic analysis

Before harvesting the implants from the peritoneum, they were counted and a vernier calliper was used to measure their length and width. Lesion size was calculated by multiplying the length and width and was expressed in mm².

Histologic studies

Tissues were fixed in 4% neutral buffered formalin overnight at 48 °C before being embedded in paraffin wax for histological and immunohistochemical analyses. Four to five non-contiguous 4 μ m sections from each specimen were stained with hematoxylin–eosin (Sigma Co.) and examined microscopically for histological hallmarks (glands and stroma) of endometriosis. Following the histological study, only the lesions that contained both glandular and stromal elements were considered to be active. Those presenting an atrophic epithelium surrounded by fibrotic tissue rather than stroma were considered non-active lesions and were discarded from the study.

Immunohistochemistry

Standard immunohistochemistry was achieved using specific primary antibodies for each determination. Proliferating cells were detected by using a mouse MAB against Ki67 (MIB-1, 1:50; DakoCytomation, Glostrup, Denmark). The blood vessel supply of the endometrial implants was determined using a rabbit polyclonal antibody against CD31 (ab28364, 1:200, Abcam, Cambridge, MA, USA), which labels endothelial cells from both micro- and macrovasculature. A mouse MAB for VEGFR2 (sc-6251, 1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and a mouse MAB for DRD2 (sc-5303, 1:50; Santa Cruz Biotechnology, Inc.) were applied to detect the

presence of these receptors in the human endometrium engraftment in the animals' peritonea.

Four micron serial sections were deparaffinized and rehydrated through graded ethanol and rinsed in distilled water. Antigen retrieval was carried out by the autoclave method (120 °C) for 3 min in 0.5 M Tris buffer at pH 10 (for CD31; Wang *et al.* 2008) or 0.01 M citrate buffer at pH 6.0 (for Ki67, VEGFR2 and DRD2) followed by cooling for 20 min at room temperature. Sections were gently rinsed in deionized water and then transferred to a 0.5 M Tris-based solution in 0.15 M NaCl with 0.1% v/v Triton-X-100 at pH 7.6 (TBST). Endogenous peroxidase was blocked for 10 min with 3% hydrogen peroxide. To further reduce non-specific background staining, slides were incubated at room temperature with an avidin/biotin blocking kit (Vector Labs, Burlingame, CA, USA). The sections were incubated overnight with the primary antibodies in a humidified chamber at 4 °C. After washing with TBST, sections were incubated with biotinylated goat anti-rabbit IgG (B8895, 1:800; Sigma–Aldrich Ltd) for CD31, or secondary link biotinylated antibody (K0679, LSAB+kit; Dakocytomation) for Ki67, VEGFR2, and DRD2 for 30 min at room temperature and then with streptavidin–HRP for 30 min, using 3,3'-diaminobenzidine as the chromogen (K0679, LSAB+kit; Dakocytomation). Sections were then counterstained with Mayer's hematoxylin and then dehydrated and mounted in mounting medium (Eukitt 15 320).

The following positive controls were used in the immunohistochemistry assay: neuroblastoma for Ki67, human breast cancer for VEGFR2, human endometrium positive for CD31, and human brain for DRD2. With the exception of ACTA2, for which a non-specific IgG antibody was used, negative controls were performed by omission and pre-absorption of the primary antibodies with a specific immunogen peptide acquired from the same company that supplied us with the primary antibodies.

Immunofluorescence

In order to determine whether vessels supporting the lesions were mature or immature, we performed a dual immunofluorescence procedure by detecting pericytes with an ACTA2 antibody (C6198, 1:500; Sigma–Aldrich Ltd) directly labeled with Cy3 in sections previously stained with CD31. We did this due to the fact that pericytes surround mature vessels but are absent in new proliferating immature vessels.

Detection of immunofluorescence was performed in the same way as immunohistochemistry, but using a Tyramide Signal Amplification Fluorescein system (NEL701A001KT, TSA; Perkin Elmer Life Sciences, Boston, MA, USA) instead of DAB, following the manufacturer's protocol. Finally, the slides were rinsed in PBS and counterstained with mounting medium ProLong Gold antifade reagent with DAPI (Invitrogen, P36931).

Morphometric analysis

High-quality images (2048×1536 pixels) from histological and immunostained sections were recorded randomly with an Olympus BH2-UMA microscope connected to a JVC/TK-1270 video camera and a computer-digitized plate (Olympus, Tokyo, Japan). The images were processed with

Image ProPlus 6.0 Software (Media Cybernetics, Silver Springs, MD, USA) in order to manually delineate regions of interest that were free of gaps and detached tissue. Subsequently, the area of interest and the stained area (to be analyzed) were manually or automatically segmented from the region of interest using the same software. Parameters of interest were estimated as follows: at least ten images of each lesion (10× field) were used to manually delimitate the glandular and stromal area and were expressed as a ratio of said area. The gland profile was achieved by counting the number of glands appearing per implant in each image (10× field) of a lesion. For every immunohistochemical parameter evaluated (except for the quantification of vessel maturity), three non-contiguous slides were photographed in four random high-power (40×) fields and processed with Image-pro plus 6.3 to automatically outline, highlight, and quantify the stained areas. The percentage of vascularization, as well as the overall distribution of DRD2 and VEGFR2, was estimated by quantifying the area stained by CD31 (endothelial cell marker), DRD2, and VEGFR2 antibodies per area of interest, according to previously described methods (Caulet *et al.* 1992, Moody *et al.* 2004). We initially aimed to evaluate the proliferation index by quantifying the number Ki67 (proliferation marker)-positive cells per total cell number in each area of interest. However, because of the diffuse (brown) staining, the software failed to automatically segment the number of brown-stained nuclei in the quantification of this parameter. Thus, we decided to evaluate proliferation by also quantifying the area stained by Ki67 per area of interest. Despite not being as accurate as counting individual proliferating cells, quantifying the Ki67-stained area is also a trustable and accepted methodology for the estimation of proliferation indexes, which has been previously used by other investigators (Caulet *et al.* 1992, Belur *et al.* 2011). Vessel maturity was determined by the operator, who manually counted the number of mature (CD31+/ACTA2+) or immature (CD31+/ACTA2–) vessels in each area of interest.

Quantitative real-time PCR

RNA extraction

Lesions destined for PCR amplification were excised and maintained at –196 °C with 100–500 µl Trizol reagent until ready for processing. The Chomczynski and Sacchi method was used for RNA extraction. The amount of mRNA was quantified in a nanodrop and its integrity assessed with an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany).

Primer design

Given that the objective of our study was to evaluate angiogenesis in a process in which both human and mouse tissues were implicated, the primers for *VEGF*, *VEGFR2*, and *Drd2* and the housekeeping gene β -actin were designed to simultaneously recognize both human and mouse transcripts. Gene sequences were obtained from GenBank (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide) and a blast search was performed to ensure that designed primers exhibited 100% homology

with both mouse and human sequences while avoiding cross alignment with other unspecific sequences. Primers were purchased from Invitrogen and Bonsai Technologies (Madrid, Spain). The oligonucleotide sequences used for amplification are summarized in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article.

RT-QF-PCR

Total RNA (1 µg) was reverse transcribed using an Advantage RT-for-PCR kit (Clontech) and the product was diluted in water to a final volume of 100 µl.

The amplification of cDNA by quantitative fluorescence (QF)-PCR was performed with the LightCycler-Fast-Start DNA Master SYBR Green I kit (Roche Diagnostics) in a Roche Light Cycler (Roche), following the manufacturer's instructions. cDNA (100 ng) was used for each sample analyzed. A calibration curve was drawn by including six serial dilutions (1/10) of a high cDNA concentration sample of each gene in order to interpolate the results in each experiment. The final products were analyzed with the software provided by the manufacturer (Roche Molecular Biochemicals Light Cycler Software v3.5). Human-mouse β -actin was used as a house-keeping gene to normalize the expression levels of *VEGF*, *VEGFR2*, and *Drd2*. Results were expressed as arbitrary fluorescence units after normalizing the ratio (gene of interest/house keeping gene) to the values obtained in the control groups (assigned value = 1).

Statistical analysis

Statistical analysis was carried out with SPSS (release 15.0 SPSS, Inc., Chicago, IL, USA). Results are expressed as mean $\pm$ S.E.M., except when specified. The Kolmogorov–Smirnov test was used to determine whether data followed a normal distribution. Non-parametric Mann–Whitney and Dunn's multiple comparisons test were used to compare individual means. The level of statistical significance was set at $P < 0.05$.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0223>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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