Dopamine agonist administration causes a reduction in endometrial implants through modulation of angiogenesis in experimentally induced endometriosis

Edurne Novella-Maestre¹,², Carmen Carda², Inmaculada Noguera³, Amparo Ruiz-Saurí², Juan Antonio García-Velasco¹, Carlos Simón¹, and Antonio Pellicer¹,⁴

¹Instituto Valenciano de Infertilidad (IVI), University of Valencia, Plaza de la Policía Local, 3, 46015 Valencia, Spain ²Department of Pathology, University of Valencia, Valencia, Spain ³Research Unit, School of Medicine and Odontology, University of Valencia, Valencia, Spain ⁴Correspondence address. E-mail: apellicer@ivi.es

BACKGROUND: Implantation of a retrogradely-shed endometrium during menstruation requires an adequate blood supply. The endometrium has angiogenic potential, and endometriotic lesions grow in areas with a rich vascularization, suggesting that angiogenesis is a prerequisite for endometriosis development. Targeting vascular endothelial growth factor (VEGF) leads to an inhibition of endometriosis. Dopamine and its agonists, such as cabergoline (Cb2), promote VEGF receptor-2 (VEGFR-2) endocytosis in endothelial cells, preventing VEGF–VEGFR-2 binding and reducing neoangiogenesis. The aim of this study was to evaluate the anti-angiogenic properties of Cb2 on growth of established endometriosis lesions and investigate the molecular mechanisms by which Cb2 exerts the anti-angiogenic effect.

METHODS: Human endometrium fragments were implanted in female nude mice peritoneum, and mice were treated with vehicle, 0.05 or 0.1 mg/kg/day oral Cb2 for 14 days. After treatment, the implants were processed to assess proliferative activity, neoangiogenesis, VEGFR-2 phosphorylation and angiogenic gene expression.

RESULTS: A significant decrease in the percentage of active endometriotic lesions (P < 0.05) and cellular proliferation index (P < 0.001) was found with Cb2 treatment. Neoangiogenesis was reduced by Cb2 treatment, as observed at gross morphological level and by significant changes in gene expression. The degree of VEGFR-2 phosphorylation was significantly lower in Cb2-treated animals than controls.

CONCLUSIONS: Cb2 treatment in experimental endometriosis has an anti-angiogenic effect acting through VEGFR-2 activation. These findings support the testing of dopamine agonists as a novel therapeutic approach to peritoneal endometriosis in humans.

Key words: endometriosis / dopamine agonists / cabergoline / angiogenesis / vascular endothelial growth factor

Introduction

Endometriosis, pathologically defined as the presence of endometrial-like tissue, glands and stroma outside the uterine cavity (Galle, 1989), is a common, estrogen-dependent disorder associated with pelvic pain and infertility. Its prevalence approaches 14% of the general population, but in women with pelvic pain, infertility or both, the frequency is 35–50% (Rawson, 1991). This disorder is most commonly diagnosed in women of reproductive age, and three main forms have been identified: peritoneal, ovarian and deep endometriosis.

Surgery commonly provides temporary relief, although endometriosis is characterized by recurrence in up to 75% of women within 2 years. Medical therapies have traditionally been used to target the estrogen dependency of the disease. They are all effective to a certain extent, and new strategies are being developed thanks to a better understanding of the disorder’s physiopathology.
Peritoneal endometriosis is believed to be the result of implantation of retrogradely-shed endometrium during menstruation, which has the capacity to adhere, attach and implant ectopically (Sampson, 1927; Maas et al., 2001a, b). An adequate blood supply is essential to endometrial survival in an ectopic location (Maas et al., 2001a, b). The endometrium has angiogenic potential and endometriotic lesions grow in areas with a constant and abundant blood supply (Nisolle et al., 1993). This suggests that angiogenesis is a prerequisite for the development of endometriosis (Nap et al., 2004).

Angiogenesis is a dynamic process involving many factors. Some pro-angiogenic factors are known to be increased in the peritoneal fluid of women with endometriosis, whereas the levels of others with anti-angiogenic properties are lower (Laschke and Menger, 2007). Vascular endothelial growth factor (VEGF), a heparin-binding glycoprotein with angiogenic and endothelial cell-specific mitogenic characteristics, and with vascular permeability (VP) properties, is considered to play a pivotal role in both physiologic and pathologic angiogenesis (Gargett et al., 2002). VEGF has been shown to be released by macrophages present in increased amounts in the peritoneal fluid of women with endometriosis (McLaren et al., 1996a, b). Moreover, there is a positive correlation between the severity of the disease and the secretion of VEGF in peritoneal fluid (Shifren et al., 2002). Vascular endothelial growth factor (VEGF-A) inhibitor (Avastin) and other angiogenesis inhibitors (TNP-470, Endostatin, Anginex) were found to significantly decrease VEGF-A expression in endometriotic implants and vascular destruction (Park et al., 2003). In a study with rats, we showed that Cabergolene (Cb2) reduced ovarian hyperpermeability and ascites formation without affecting corpus luteum angiogenesis and function, thus exerting a dose-related dual effect. This effect was shown to be mediated by dephosphorylation of the VEGFR-2 (Gómez et al., 2006). Ascites, hemoconcentration and ovarian perfusion were also reduced in human beings (Álvarez et al., 2007). Cb2 is currently used, for example, for the suppression of breast-feeding and treatment of hyperprolactinaemia (Gillam et al., 2006; Colao et al., 2007; Buhendwa et al., 2008). Experience with the use of Cb2 in pregnancy shows that there is no increased risk of spontaneous miscarriage, premature delivery, multiple pregnancy or congenital abnormalities (Robert et al., 1996; Ricci et al., 2002). Short-term follow-up studies of infants born to mothers who used Cb2 during pregnancy indicate normal neonatal physical and mental development (Robert et al., 1996).

Based on the aforementioned findings, we developed an experimental endometriosis model in which human endometrial sections were implanted into nude mice. Our initial hypothesis was that administration of Cb2 would decrease angiogenesis and affect the overall structure of these implants through inactivation of the VEGFR-2.

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 employing 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 performed as previously described (Nap et al., 2004) with minor modifications. A total of 60 ovariectomized 5-week-old female mice (Hsd: athymic nude, Harlan Ibérica S.L., Barcelona, Spain) were individually housed in autoclaved cages and bedding, in laminar flow filtered hoods. The animal room was maintained at 26 °C with a 12-h light, 12-h dark cycle, and mice were fed ad libitum with autoclaved laboratory rodent Chow and acidified water. All handling was performed in laminar flow filtered hoods. A mixture of ketamine (Ketolar 10 mg/g ketamine and 1 g/g medetomidine) injected i.p. was used to anaesthetize mice before invasive procedures and atipemazol (Antisedan 2 mg/g keta
damine) injected i.p. to reverse the anaesthesia effects, was used after invasive procedures using sterile instruments. Sixty-day release sterile capsules containing 18 mg 17β-estradiol (E2) (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 from 20 oocyte donors (18–34 years old) at ovum retrieval with normal menstrual cycles and no history of endometriosis, and were fixed in the peritoneum of each mouse using n-butyl-ester cyanoacrylate adhesive (3M Animal Care products). Human endometrial samples from each individual donor were used in two animals of each

related angiogenesis and VP in a mouse cancer model by interfering with VEGF/VEGFR-2 signalling (Basu et al., 2001). In vitro studies have suggested that the molecular mechanism underlying this action involves the internalization of VEGF-2 induced by the activation of the Dp-r2 (Basu et al., 2001).

We have also gathered information by employing dopamine agonist (DA) in the prevention of ovulation stimulation syndrome in both animals (Gómez et al., 2006) and humans (Álvarez et al., 2007). In a study with rats, we showed that Cabergolene (Cembrance) reduced ovarian hyperpermeability and ascites formation without affecting corpus luteum angiogenesis and function, thus exerting a dose-related dual effect. This effect was shown to be mediated by dephosphorylation of the VEGFR-2 (Gómez et al., 2006). Ascites, hemoconcentration and ovarian perfusion were also reduced in human beings (Álvarez et al., 2007). Cb2 is currently used, for example, for the suppression of breast-feeding and treatment of hyperprolactinaemia (Gillam et al., 2006; Colao et al., 2007; Buhendwa et al., 2008). Experience with the use of Cb2 in pregnancy shows that there is no increased risk of spontaneous miscarriage, premature delivery, multiple pregnancy or congenital abnormalities (Robert et al., 1996; Ricci et al., 2002). Short-term follow-up studies of infants born to mothers who used Cb2 during pregnancy indicate normal neonatal physical and mental development (Robert et al., 1996).

Based on the aforementioned findings, we developed an experimental endometriosis model in which human endometrial sections were implanted into nude mice. Our initial hypothesis was that administration of Cb2 would decrease angiogenesis and affect the overall structure of these implants through inactivation of the VEGFR-2.
The lesions were fixed in 4% neutral buffered formalin overnight at 4°C before being routinely paraffin wax embedded and cut at 4 μm serial sections. Four to five non-contiguous sections from each specimen were stained with haematoxylin–eosin (H&E) and examined microscopically for the histological hallmarks (glands and stroma) of endometriosis. After the histological study, only the lesions that contained both glandular and stromal elements were considered active lesions. The lesions were fixed in 4% neutral buffered formalin overnight at 4°C before being routinely paraffin wax embedded and cut at 4 μm serial sections. Four to five non-contiguous sections from each specimen were stained with haematoxylin–eosin (H&E) and examined microscopically for the histological hallmarks (glands and stroma) of endometriosis. After the histological study, only the lesions that contained both glandular and stromal elements were considered active lesions. The lesions were fixed in 4% neutral buffered formalin overnight at 4°C before being routinely paraffin wax embedded and cut at 4 μm serial sections. Four to five non-contiguous sections from each specimen were stained with haematoxylin–eosin (H&E) and examined microscopically for the histological hallmarks (glands and stroma) of endometriosis. After the histological study, only the lesions that contained both glandular and stromal elements were considered active lesions.
Statistical analysis
Statistical analyses were performed using GraphPad Instat V3.0 (GraphPad Software, San Diego, CA, USA). Kruskal–Wallis test was employed for the overall analysis of the data to determine if they followed a normal distribution. Non-parametric Mann–Whitney and Dunn’s multiple comparisons test were used to compare individual means. Categorical data were expressed as number and percentage, and numerical data as mean ± SEM, except when specified. In all cases, significance was considered to be P < 0.05.

Results
Evaluation of the endometriosis model
A total of 3 out of 60 animals, in which the endometrium of the same donor was implanted, died during the experimental procedures prior to Cb2 administration, due to an infection during the ovariectomy surgery. Macroscopic lesions were observed on the peritoneal wall in all experimental groups. In order to test the effectiveness of the model, we compared the number of implants found in the peritoneum of control group mice before and after treatment. Of the four endometrial implants introduced per animal, a mean of 2.8 ± 0.2 were detected (70.8 ± 5.2% recovery rate), of which, after histological analysis, 2.5 ± 0.3 (89.6 ± 5.7%) were considered to be active lesions composed of glands and stroma.

Regression of lesions after Cb2 treatment
In all groups three to four macroscopic lesions were recovered. After the histological study, combined with morphometric techniques, we found that the percentage of recovered active lesions was significantly (P < 0.05) decreased when low- (58.6 ± 9.7%) or high- (60.4 ± 8.4%) dose Cb2 was employed as compared with the controls (89.6 ± 5.7%) (Fig. 1A). Moreover, endometriotic lesions in the controls presented a high cellular stroma and histological aspect of complete reorganization. However, in Cb2-treated lesions, we observed a lax stroma with a lack of cellularity and organization, which is characteristic of an atrophic or degenerative tissue (Fig. 1B).

These results were confirmed by morphometric analysis. There were no significant differences (P = 0.86) in the lesion size between the control (0.032 ± 0.01 mm²), low (0.032 ± 0.01 mm²) and high (0.034 ± 0.01 mm²) doses group. However, the glands/stroma ratio was significantly (P < 0.05) lower in mice treated with low (0.33) and high (0.35) doses of Cb2 as compared with controls (0.52); while the stroma area was significantly higher, a small proportion of glands area was quantified in the groups treated with Cb2, demonstrating that treatment produced a decrease in the number of endometrial glands.

Cb2 treatment affects cell proliferation
The proliferative status of endometriotic cells was investigated using Ki-67 labelling index, where the total antigen expression per square micrometer (proliferation index) was quantified by morphometric analysis. We observed a lower level of Ki-67 staining of nuclei in the glands and stroma of Cb2-treated animals than in controls (Fig. 2A). When the proliferative index was calculated, it was significantly (P < 0.001) lower in mice treated with low (0.02 ± 0.01) and high (0.03 ± 0.01) doses of Cb2 than in control animals (0.12 ± 0.02) (Fig. 2B).

Cb2 treatment impairs neoangiogenesis
When the peritoneal cavity was opened, a rich vascular net was observed in the endometriotic lesions of the control animals, while those of Cb2-treated animals were of a whitish colour and revealed a less well-developed vascularization (Fig. 3A).

To test the anti-angiogenic action of Cb2, a double immunostaining and laser scanning confocal microscopy analysis were performed, employing antibodies against VWF and αSMA (Fig. 3B). The blood vessels that expressed only VWF (vWF+/αSMA−) were considered new or immature, while the blood vessels surrounded by αSMA (vWF+/αSMA+) were classified as old or mature.

There was a significant difference (P < 0.001) among the groups with respect to the newly formed and mature blood vessels. The formation of new blood vessels was suppressed in endometriosis lesions in mice treated with low (13.5 ± 1.1%) and high (10.8 ± 3.2%) doses of Cb2 as compared with controls (75.4 ± 1.6%) (Fig. 3C).

Vascular density (VD), determined on the basis of VWF+ vessels per square millimetre, was also calculated. There was no statistical difference among the groups in the VD (P > 0.05) (Fig. 3D).

Cb2 modulates the expression of genes related to angiogenesis in lesions
Employing quantitative RT–PCR TaqMan technology, several molecular pro-angiogenic markers were analysed. VEGF expression was significantly (P < 0.05) lower in lesions treated with low (0.5 ± 0.2) and high (0.4 ± 0.1) doses of Cb2 than in controls (1.2 ± 0.3) (Fig. 4A). Similarly, Notch-4 expression was significantly (P < 0.05) down-regulated in the lesions treated with high doses of Cb2 as compared with controls (0.4 ± 0.1) (Fig. 4B).

In addition, we studied the anti-angiogenic genes Ang-1 and Wnt.

The expression of Ang-1 was significantly (P < 0.05) up-regulated in the lesions of mice treated with high doses of Cb2 (3.7 ± 0.6) compared with controls (1.1 ± 0.2) (Fig. 4C). Wnt-1 expression tended to be higher in the lesions of both groups of Cb2-treated mice than in controls (P = 0.07) (Fig. 4D).

Cb2 anti-angiogenic action is mediated by inhibition of VEGFR-2 phosphorylation
We studied total VEGFR-2 expression and the phosphorylation state of VEGFR-2 tyrosine 951 (pVEGFR-2), the first phosphorylation site for the transduction of ligand-dependent VEGFR-2 signalling, by employing immunohistochemistry and morphometry assays. The VEGFR-2 activity in blood vessels was expressed as (pVEGFR-2/total VEGFR-2) × 100.

The VEGFR-2 was expressed in the endometrial glandular epithelium and stromal cells, as well as in the endometrial blood vessels. A decrease of the total VEGFR-2 and pVEGFR-2 staining in blood vessels of the Cb2-treated groups with respect to that of controls was observed (Fig. 5A). Morphometric analysis revealed that Cb2 administration at low (12.5 ± 1.0) and high (8.3 ± 1.6) doses reduced VEGFR-2 activity with regard to that in controls (66.86 ± 1.9) by as much as 87 and 81%, respectively (Fig. 5B).
Discussion

The results of the present study demonstrate that the treatment of nude mice, in which human endometrial tissue was implanted, with two different doses of Cb2 produces a significant decrease in the percentage of active lesions 2 weeks after initiation of treatment. Moreover, the ratio glands/stroma was significantly lower after Cb2 administration, and lax stroma with lost cellularity and organization was also a feature of animals treated with Cb2. Both are characteristic of atrophic or degenerative tissue. Similarly, a significant decrease in the proliferation index, as ascertained by Ki-67 staining, provided further confirmation of the detrimental effects of Cb2 on these tissues.

The action of Cb2 on the experimental lesions was directed through the angiogenic process. First, we observed, at a macroscopic level, a rich vascular net in the controls, which confirmed that the implants were active due to the positive action of the E2 pellets. The lesions of the animals treated with Cb2, however, presented a white aspect and a less developed vascularization. Similarly, the microscopic findings revealed areas of active necrotic processes in the Cb2-treated mice, due to the inhibition of new vascular nets. Immunohistochemistry and confocal microscopy showed that there was no significant difference

Figure 1 Microscopic appearance of the lesions (haematoxylin–eosin staining). Twelve animals were included per group in three different experiments. (A) Percentage of active lesions showing both glands and stroma in the peritoneum of the three experimental groups (mean ± SEM). There was a significant decrease in the percentage of active lesions in both cabergoline (Cb2)-treated groups as compared with controls (P < 0.05). (B) Microphotographs (original magnification ×100 and ×200) showing a lax stroma with a lack of cellularity and organization, which is characteristic of an atrophic or degenerative tissue in Cb2-treated animals.
among the groups in terms of VD. An explanation for this finding could be that in the model employed, revascularization starts 5–8 days after implantation and involves the disappearance of native graft vessels, coinciding with the invasion of the interface and then the stroma by murine vessels (Eggermont et al., 2005). Thus, the anti-angiogenic action of DA may be inhibiting the revascularization process. It is important to mention that due to the revascularization process, it was necessary to employ antibodies which detected both human and murine blood vessels markers, in order to be able to analyse the DA anti-angiogenic effect (except in the case of Ki-67 antibody used to analyse the proliferation index in human endometrial implants). The neoangiogenesis study showed that there were significantly more newly formed vessels in control animals than in the treated groups. Established vessels are characterized by a covering of αSMA positive pericytes, which renders them less sensitive to VEGF (Benjamin et al., 1998). Pericyte-free vessels are very sensitive to the angiogenic signals or vasoactive elements, growing in the presence of VEGF and undergoing apoptosis in its absence. Thus, the high number of immature pericyte-free vessels in control lesions could be indicative of the progression of the human endometrium invasion by murine blood vessels, while the high quantity of mature pericytes vessels in Cb2-treated lesions could be indicative of the anti-angiogenic effect of DA.

We also analysed the angiogenic process at a molecular level. In this sense, the expression of pro-angiogenic markers such as VEGF and VEGFR-2 was found to be significantly lower in Cb2-treated mice than in controls. Although expression of Notch-4 was not significantly different, a clear trend towards decreased expression was certainly evident when treatment included Cb2. In contrast, the expression of Ang-1, an anti-angiogenic marker, was significantly enhanced after Cb2 administration, and a trend towards increased Wnt-1 expression was also observed in the two Cb2-treated groups.

To investigate the mechanism of Cb2 action, we focused on the VEGF/VEGFR-2 signalling pathway, since evidence has been acquired that demonstrates that DA bind to their type-2 receptor and inactivate the VEGFR-2, thereby avoiding its phosphorylation (Gómez et al., 2006). In these studies, we confirmed that the degree of VEGFR-2 phosphorylation was significantly lower in Cb2-treated mice than in controls.

Although we have focused our research on Dp and its effects on the VEGF system, it is worth noting that DA may exert their action...
Figure 3  Analysis of neoangiogenesis in the three groups. Twelve animals were included per group in three different experiments. (A) Macroscopic evaluation of endometriosis lesions after treatment shows a less well-developed vascular net in the lesions from mice treated with low and high doses of Cb2 than in controls. (B) The neoangiogenesis study shows that not all von Willebrand Factor (vWF) positive vessels (red, arrow) were α-smooth muscle actin (αSMA) positive (green), indicating that both mature and newly formed vessels were present in these lesions; in Cb2-treated lesions, most of the blood vessels were both vWF and α-SMA positive, indicating inhibition of the angiogenic process. (C) Animals treated with low and high doses of Cb2 had significantly more mature and fewer newly formed blood vessels than controls (mean ± SEM) (*P < 0.001). (D) Vascular density (VD) was similar in the lesions of all three groups.
through inhibition of prolactin (PRL) secretion. PRL is a powerful angiogenic inducer, and regulates decidualization and breast growth during pregnancy (Reese et al., 2000). Experimentally, PRL induces angiogenesis in other tissues, such as muscle. PRL exerts its pro-angiogenic effect through blood vessel receptors. Thus, employing DA and reducing serum PRL levels may constitute a parallel mechanism for the effects of Cb2 in this animal model. Interestingly, several reports have focused on the fact that hyperprolactinaemia is associated with the presence of endometriosis (Gregoriou et al., 1999; Cunha-Filho et al., 2002). Authors believe that this hyperprolactinaemic state could explain infertility related to mild and moderate endometriosis, but perhaps they have missed the main point: hyperprolactinaemia may increase angiogenesis and induce/maintain endometriotic lesions.

Our approach, by which we target VEGF to decrease angiogenesis and reduce the extent and activity of endometriotic lesions, is similar to others previously employed in experimental models (Hull et al., 2003; Nap et al., 2004). In addition to some methodological changes, the main difference between our study and those previously mentioned is that we chose a well-established medication that is widely used for hyperprolactinaemic states, and which has far fewer side effects than other anti-VEGF drugs.

Two other issues deserve consideration. First, we have to acknowledge the limitations of the model. These mice do not spontaneously develop endometriosis and also they are immune compromised and endometriosis has an important immune component. However, endometriosis is a difficult disease to study and we firmly believe that these results provide the rationale for a pilot study in humans because we showed that ectopic endometrium can be targeted successfully.

Second, the safety of DA should be considered. Although it was not the purpose of this study to test toxicity in the nude mice employed as an experimental model, it is of relevance that none of them died after initiation of Cb2 treatment, and that they did not experience weight loss with respect to controls (data not shown). Whether DA may affect angiogenesis in the reproductive organs was not tested in these experiments, but we showed in previous experiments that, using 50 and 100 μg/kg/day, angiogenesis and corpus luteum function were unaffected (Gómez et al., 2006). It is worth mentioning that lower doses of Cb2 were at least as effective as higher doses. Thus, an issue to be explored in the future is the lowest effective dose in order to counteract possible side effects of higher doses.

If these experiments are the basis of future clinical trials, the issue of possible side effects should be analysed in detail, since the potential benefits of DA in the treatment of endometriosis could be hampered by impairment of implantation in women who frequently try to become pregnant. The dual effects on ectopic and eutopic endometrium may also be highly dependant on the doses employed in humans since it is well known that women receiving high doses of Cb2 become pregnant uneventfully (Verhelst et al., 1999). But even if both types of endometria are affected by this new use of DA, clinical trials should be set up to target ectopic tissue because a medical treatment of endometriosis is needed and the medications available to date also interfere with pregnancy.

Moreover, recently the use of Cb2 and pergolide (another DA) for the treatment of chronic conditions, such as Parkinson’s disease, hyperprolactinaemia and the restless leg syndrome, has been consistently associated with an increased incidence of cardiac valve

**Figure 4** Pro- and anti-angiogenic markers were analysed in lesions using quantitative RT–PCR TaqMan technology. Twelve animals were included per group in two different experiments but because some animals died before Cb2 administration, seven animals were used for gene expression studies. (A) Vascular endothelial growth factor (VEGF) expression was significantly (*P < 0.05) lower in lesions from mice treated with low and high Cb2 compared with controls. (B) Notch-4 expression was significantly (*P < 0.05) down-regulated in the lesions from mice treated with high doses of Cb2 as compared with controls. (C) Expression of angiopoietin-1 (Ang-1) was significantly (P < 0.05) up-regulated in the lesions from mice treated with high doses of Cb2 compared with controls. (D) Wnt-1 expression tended to be higher in the lesions of both groups of Cb2-treated mice than in controls (P = 0.07) (mean ± SEM).
regurgitation (Schade et al., 2007; Zanettini et al., 2007). In the context of endometriosis, it would be important to explore the use of other DAs, which may not carry the same side effects profile. In fact, our group is already carrying out such work.

Taking our findings and the evidence from the literature into account, we firmly believe that the use of DA will be successful in the treatment of peritoneal endometriosis. However, the most appropriate DA for a chronic disease, such as endometriosis, should be selected to avoid the above-mentioned side effects.

Acknowledgements

The authors acknowledge the assistance of Alejo Sempere and Samuel Navarro (Department of Pathology, University of Valencia), Angel Ortega (Department of Physiology, University of Valencia), Nicolas Garrido (IU-IVI-UV), Pilar Oviedo (Department of Obstetrics Gynaecology, University of Valencia) and Francisco Dasi (Department of Pharmacology, University of Valencia) for their teaching and advice.

Eva Sanchez Gomez (National Stem Cell Bank, Centro de Investigación Principe Felipe) and Julio Rosa da Silva are also acknowledged for the excellent technical assistance in cell culture. We especially thank Lina March, Maria Sanchez-Serrano, Svend Lindenberg and Mette Munk for their critical input and corrections of the manuscript.

Funding

This work was supported by SAF2007-65334 grant from the Spanish Government and Lilly Foundation Grant for Research in Clinical Medicine.

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Submitted on August 8, 2008; resubmitted on December 15, 2008; accepted on December 28, 2008