microRNAs related to angiogenesis are dysregulated in endometrioid endometrial cancer

Luis A. Ramón1,†, Aitana Braza-Boils1,†, Juan Gilabert2, Melitina Chirivella3, Francisco España1, Amparo Estellés1, and Juan Gilabert-Estellés4,*

1Research Center Anatomopathology Department, Hospital Universitario y Politécnico La Fe, Valencia, Spain 2Gynecology Service, Hospital Arnau de Vilanova, Valencia, Spain 3Anatomopathology Department, Hospital Universitario y Politécnico La Fe, Valencia, Spain 4Instituto Maternoinfantil, Hospital General Universitario, Valencia, Spain

*Correspondence address. Tel: +34-600771953; Fax: +34-961973018; E-mail: juangilaeste@yahoo.es

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STUDY QUESTION: Which is the role of microRNAs (miRNAs) related to several angiogenesis regulators such as VEGF-A (Vascular endothelial growth factor-A) and TSP-1 (Thrombospondin-I) in endometrial cancer?

SUMMARY ANSWER: A dysregulated expression of miRNAs related to angiogenesis and an increase in the VEGF-A levels were observed in endometrial cancer in comparison with control. The different expression of miRNAs could modulate the expression of angiogenic and antiangiogenic factors, which may play an important role in the pathogenesis of endometrial cancer.

WHAT IS KNOWN ALREADY: Dysregulated miRNA expression has been previously evaluated in endometrial adenocarcinoma. To the best of our knowledge, there are no studies on the relationship between angiogenic factors and miRNAs in endometrial cancer.

STUDY DESIGN, SIZE, DURATION: Case–control study: 41 patients with histologically proven endometrioid endometrial cancer and 56 women without endometrial cancer.

PARTICIPANTS/MATERIALS, SETTING, METHODS: RNAs isolated from tissue samples were analyzed using the GeneChip miRNA 2.0 Array platform (Affymetrix). TaqMan qRT–PCR was used to assess the expression of the selected miRNAs related to angiogenesis (miR-15b, -16, -17-5p, -20a, -125a, -214*, -221, -222 and -424), and VEGF-A and TSP-1 mRNAs were assessed by qRT–PCR using SYBR Green. Protein levels were quantified by ELISAs.

MAIN RESULTS AND THE ROLE OF CHANCE: Compared with the miRNAs in the control endometrium, eight miRNAs (miR-15b, -17-5p, -20a, -125a, -214*, -221, -222, and -424) were significantly down-regulated and two miRNAs (miR-200b and -210) were significantly up-regulated in the cancerous endometrium. A significant increase in VEGF-A mRNA and protein expression and in TSP-1 protein levels (P < 0.01) was observed in endometrial cancer. Moreover, significant inverse correlations between VEGF-A protein levels and miR-20a, -125a, -214*, -221, -222 and -424 were detected. In contrast, a positive correlation was observed between VEGF-A and miR-200b and -210. Furthermore, stage IB endometrial cancer was associated with a higher VEGF-A protein/mRNA ratio and lower miR-214*, -221, and -222 expression in comparison with stage IA.

LIMITATIONS, REASONS FOR CAUTION: Future functional studies (e.g. miRNA inhibition or ectopic overexpression) in cell culture models are needed to confirm the VEGF targeting by the miRNAs found in the present study.

WIDER IMPLICATIONS OF THE FINDINGS: The findings of the present study have potential implications for diagnostics and therapeutics of endometrial carcinoma.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by research grants from the Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica (Instituto de Salud Carlos III, Fondo de Investigación Sanitaria, PI080185, PI0110091)

† L.A.R. and A.B.-B. have contributed similarly in the present article.
Introduction

Endometrial cancer is one of the most prevalent malignancies in developed countries (Jemal et al., 2010). However, the pathological mechanisms underlying the development of endometrial cancer and metastasis are among the least studies of the gynecological malignancies (Devor et al., 2011). Gene expression profiling studies have demonstrated that many genes are dysregulated in cancer. Recently, it has been described that microRNAs (miRNAs) are dysregulated in several human cancers (Boren et al., 2008; Le et al., 2010; Ratner et al., 2010; Gilabert-Estelles et al., 2012). miRNAs are 19–25 nucleotide non-coding RNAs that regulate gene expression and play fundamental roles in biological processes. These small molecules bind to target mRNAs and function as post-transcriptional gene regulators, mediating translational repression and/or mRNA degradation (Bartel, 2004, 2009). Functional analysis of miRNAs has revealed their significant regulatory influence on the translation of target genes involved in both physiological and pathological conditions (Ambros, 2004; Kuokkanen et al., 2010). Importantly, miRNA expression is cell-type-specific; each miRNA is able to target hundreds of mRNAs, and large numbers of miRNA target the same mRNA.

Abnormal miRNA expression is associated with several human diseases, including cancer, cardiovascular disorders, inflammatory diseases and benign or malignant pathologies of the female reproductive tract (Ohlsson Teague et al., 2009; Sonkoly and Pivarcsi, 2009; Zorio et al., 2009; Ramón et al., 2011; Gilabert-Estelles et al., 2012). In fact, specific miRNAs may act as biomarkers of the outcome for that cancer type (Le et al., 2010; Ratner et al., 2010; Lee et al., 2011). Because alterations in miRNA expression are involved in the initiation, progression and metastasis of human cancer, miRNAs may function as tumor suppressors or oncogenes in cancer development (Nicoloso et al., 2009; Shenouda and Alahari, 2009; Lam et al., 2012). In addition, because angiogenesis is important to the pathogenesis of cancers, miRNAs, which may be the main regulators of angiogenesis (Fish and Srivastava, 2009; Suarez and Sessa, 2009), may play a role in cancer progression.

There is consistent evidence that vascular endothelial growth factor (VEGF) plays a central role in disease progression and prognosis of gynecological cancers, including endometrial cancer (Kamat et al., 2007). VEGF-A represents one of the most potent angiogenic factors (McLaren, 2000; Carmeliet, 2003). Thrombospondin-1 (TSP-1) is an inhibitor of angiogenesis; it has been reported that alterations in TSP-1 expression may be involved in many diseases of the reproductive tract, in which vessel formation occurs (Kawano et al., 2005; Gilabert-Estelés et al., 2007).

Dysregulated miRNA expression has been previously evaluated in endometrial adenocarcinoma (Boren et al., 2008; Chung et al., 2009; Cohn et al., 2010; Ratner et al., 2010; Gilabert-Estelles et al., 2012). Moreover, many studies have described the relationship between miRNAs and angiogenesis. miRNAs have a bidirectional implication in angiogenesis. On one hand, miRNAs may target genes involved in angiogenesis; on the other hand, miRNA expression may be modulated by angiogenic or antiangiogenic stimuli (Wang and Olson, 2009; Caporali and Emanueli, 2011).

In this study, we compared the miRNA profiles of the control endometrium with those of endometrioid endometrial cancer. Of those miRNAs with a significantly altered expression in endometrial cancer, we selected eight miRNAs involved in angiogenesis processes. We also selected four that did not show significant alterations, since several studies implicate them as important miRNAs related to angiogenesis in other gynecological diseases (Wang and Olson, 2009; Ramón et al., 2011; Gilabert-Estelles et al., 2012).

Moreover, we also studied the expressions of angiogenic (VEGF-A) and antiangiogenic (TSP-1) factors. To the best of our knowledge, there are no studies on the relationship between angiogenic factors and miRNAs in endometrial cancer.

The aim of this study was to describe the different miRNA expression profiles in endometrioid endometrial cancer and to analyze the expression of several miRNAs involved in angiogenesis and the expression of the main angiogenic (VEGF-A) and antiangiogenic (TSP-1) factors in endometrial cancer. We also analyzed the association of the studied parameters with tumor severity in women with endometrial cancer.

Materials and Methods

Clinical groups

We evaluated 41 patients with histologically proven endometrioid endometrial cancer (mean age 61 years; range 39–79 years). The tumor characteristics are recorded in Table I. All patients underwent systematic examination of the abdominal cavity and total hysterectomy with bilateral salpingo-oophorectomy. Pelvic and para-aortic lymphadenectomy were performed for undifferentiated tumors or in the presence of infiltration of the outer half of the myometrium. Endometrial carcinoma was classified following the revised International Federation of Gynecology and Obstetrics (FIGO) staging classification for gynecological oncology (Pecorelli, 2009). The tumor grade, including the architectural and nuclear features, was determined as described by Kurman et al. (1994). The mean follow-up period for all patients was 28.35 months (range 3–132 months).

Fresh frozen endometrial biopsies were obtained by aspiration from 56 women without endometrial cancer who underwent surgery for pelvic pain (16%) or tubal sterilization (84%) (mean age 43 years; range 34–48 years). The absence of other gynecologic pathologies such as endometriosis, adnexal masses or pelvic masses was confirmed in this control group by preoperative gynecologic ultrasound and systematic laparoscopic examination of the abdominal cavity during the intervention.

None of the women had received hormonal treatment for at least 3 months prior to study initiation. All participants gave informed consent...
for the study; additional consent was obtained from women who supplied tissue samples. The study was approved by the Institutional Review Board.

**Tissue extracts**

All samples were rinsed in sterile phosphate-buffered saline (Dulbecco’s; Gibco BRL, Life Technologies Ltd, Paisley, UK) and frozen in liquid nitrogen until used. Cytosolic and membrane extracts from endometrial tissues were obtained as described previously (Gilabert-Estellés et al., 2003). Total protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL, USA).

**RNA extraction and quality determination**

Total RNA was extracted from endometrial cancer and control endometrial tissues using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA), according to the manufacturer’s protocol. The RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). An OD260/280 ratio of higher than 1.8 was assumed to be an indication of good RNA purity, whereby making it suitable for measuring gene expression. RNA integrity was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). We established an RNA Integrity Number cutoff value of ≥7.0 for microarray assays; this was based on the definition used by Libberson et al. (2009) to define good RNA quality.

**miRNA expression profile study**

For each sample, 500 ng of total RNA was labeled with the FlashTag Biotin RNA Labeling Kit for Affymetrix GeneChip miRNA 2.0 arrays (Genisphere, Hatfield, PA, USA) according to the manufacturer’s recommendations. Briefly, a tailing reaction was carried out at 37°C for 15 min in a volume of 15-μl reaction mix (10 μl of total RNA and spike control oligos mix, 1 reaction buffer, 2.5 mM MnCl₂, ATP and 1 μl poly A polymerase enzyme) followed by the ligation of the biotinylated signal molecule to the target RNA sample at 25°C for 30 min (with the addition of 4 μl of 5× FlashTag Ligation Mix Biotin and 2 μl of T4 DNA Ligase into the 15 μl of reaction mix). Finally, 2.5 μl of stop solution was added to stop the reaction.

After labeling, the samples were hybridized on Affymetrix GeneChip miRNA 2.0 arrays (Affymetrix, Santa Clara, CA, USA) at 48°C and 60 rpm for 16 h in a volume of 100-μl reaction mix (which contains 20 μl of total biotin-labeled RNA, hybridization mix, formamide, DMSO, eukaryotic hybridization controls and control iligonucleotide B2). Immediately following hybridization, the arrays were washed and stained with streptavidin phycoerythrin conjugate in the GeneChip® Fluidics Station 450. Finally, they were scanned on a GeneChip® Scanner 3000 7 G.

**Microarray data analysis**

For data analysis, the .cel data files were imported into PARTEK Genomic Suite software (PARTEK, St Louis, MO, USA) and normalized using the robust multiarray analysis algorithm for background adjustment and log2-transformation. After statistical analysis, PARTEK created miRNA lists that included only miRNAs with P-values of <0.05 (cancer versus control) in one-way analysis of variance.

PARTEK also performs the principal component analysis (PCA). PCA was applied to all samples that were characterized by miRNA expression on Affymetrix GeneChip miRNA 2.0. Hierarchical cluster analysis of differentially expressed miRNA from all studied samples was performed by PARTEK to identify samples with similar patterns of miRNA expression. Compared with PCA, hierarchical clustering uses only miRNAs that are differentially expressed.

RNU6B was used as an endogenous control in the miRNA expression studies. RNU6B was present on the microarray and found to be stably expressed over all samples.

**Identification of miRNA target genes by computational tools**

PARTEK designs a list of target genes that could be regulated by those differentially expressed miRNA within samples. To corroborate results obtained from array by real-time qRT-PCR, we selected those differentially expressed miRNAs whose targets were involved in angiogenesis and those that did not show differential expression, but are known to be involved in other gynecological diseases and/or cancer.

**Quantification of selected mature miRNAs by quantitative real-time RT–PCR**

For this study, we selected a set of 12 miRNAs related to angiogenesis (miR-15b, -16, -17-5p, -20a, -21, -125a, -200b, -210, -214*, -221, -222 and -424) and RNU6B (small-nuclear RNA) that was employed as an endogenous control.

Quantification using the TaqMan® MicroRNA Assays was performed by a two-step qRT–PCR of total RNA samples. This method quantifies exclusively mature miRNAs but not their precursors.

In the RT step, cDNA was reverse transcribed from total RNA samples by using specific miRNA primers from the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Each RT reaction consisted of 7.3 μl master mix, 2 μl 5× TaqMan® MicroRNA RT primer and 0.7 μl 10 ng/μl dilution of total RNA. The Master Mix contained 10× RT buffer, 0.25 mM of each dNTP, 3.33 U/μl of MultiScribe reverse transcriptase and 0.67 U/μl of RNase inhibitor. The reactions were performed in an Eppendorf Mastercycler Thermocycler for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then maintained at 4°C.

In the PCR step, cDNA samples were amplified in duplicate with 1.5 μl cDNA, 10 μl 2× TaqMan® Universal PCR Master Mix, 1 μl of 20× TaqMan® miRNA assay [containing a mix of miRNA-specific forward PCR primer, a specific reverse PCR primer and an miRNA-specific TaqMan® MGB probe (Applied Biosystems)] and water to make a total of 20-μl reaction volume. PCRs were performed using a 7300 real-time PCR system (Applied Biosystems). After denaturing the cDNA and activating the AmpliTaq Gold® Enzyme polymerase for 10 min at 95°C, the cycling conditions were as follows: 40 cycles consisting of denaturation at 95°C for 15 s and then annealing/extension at 60°C for 60 s. Relative

**Table I Tumor characteristics**

<table>
<thead>
<tr>
<th>Histology</th>
<th>Number (%)</th>
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<tbody>
<tr>
<td>Endometrioid</td>
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</tr>
<tr>
<td>Grading</td>
<td></td>
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<tr>
<td>G1</td>
<td>28 (68.3)</td>
</tr>
<tr>
<td>G2</td>
<td>12 (29.3)</td>
</tr>
<tr>
<td>G3</td>
<td>1 ( 2.4)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>29 (70.7)</td>
</tr>
<tr>
<td>IA</td>
<td>21 (51.2)</td>
</tr>
<tr>
<td>IB</td>
<td>7 (19.5)</td>
</tr>
<tr>
<td>II</td>
<td>7 (17.1)</td>
</tr>
<tr>
<td>III</td>
<td>5 (12.2)</td>
</tr>
</tbody>
</table>
Quantification of miRNA expression was calculated using the $2^{-\Delta \Delta Ct}$ method (Livak and Schmittgen, 2001), and data were normalized to the endogenous control RNU6B.

**Quantification of mRNAs by quantitative real-time RT–PCR**

One microgram of total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into first-strand cDNA by using Superscript RNase H<sup>-</sup> (Invitrogen) with an oligo-(dT)<sub>15</sub> primer (Promega, Madison, WI, USA). cDNA was stored at $-20^\circ C$ until use.

Analysis of VEGF-A, TSP-1 and β-actin (control gene) mRNA expression was performed in a LightCycler apparatus, using version 3.5 software (Roche Molecular Biochemicals, Mannheim, Germany).

The specific primers used for the amplification of VEGF-A, TSP-1 and β-actin, the reaction mixture and the PCR conditions were as described previously (Gilabert-Estellés et al., 2007).

**VEGF-A and TSP-1 protein quantification**

The VEGF-A protein level was measured using a commercially available ELISA kit (Human VEGF, IBL International, Germany). No cross-reactivity or interference with platelet-derived growth factor was observed. This assay recognizes human VEGF-A<sub>165</sub> and VEGF-A<sub>121</sub> isoforms. The intra- and inter-assay variabilities were 4–6 and 7–10%, respectively.

The TSP-1 protein level was quantified using a commercially available ELISA kit (Human TSP-1, ELISA Development System, DuoSet, RD systems, Minneapolis, MN, USA). No cross-reactivity or interference with TSP-2 or TSP-4 was observed. The intra- and inter-assay variabilities were 5–6 and 8–11%, respectively.

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**Figure 1** Clustering analyses of tissue samples from endometrial cancer and control endometrium. **(A)** PCA of samples. PCA was applied to the samples that were characterized by the gene expression of all probes on the Affymetrix GeneChip miRNA 2.0 array platform. **(B)** Hierarchical clustering analysis of endometrial cancer and control endometrium samples.
**Statistical analysis**

All variables were checked for normal distribution with the Kolmogorov–Smirnov test. All the studied parameters showed a normal distribution. Differences between the studied variables in the tissue extracts from endometrial cancer and control endometrium were analyzed by Student’s t-test. miRNA expression relative to RNU6B was calculated and the results were normalized so that the average relative expression in the controls became 1. Values are expressed as the mean ± standard error of the mean (SEM). The levels of significance in correlations between variables were calculated using the bivariate Pearson correlation test. *P-values of <0.05 (two-tailed) were considered significant. All tests were performed using the statistical package SPSS Release 11.5 for Windows (SPSS Inc.).

**Results**

**miRNA microarray expression profiles**

The GeneChip miRNA 2.0 Array contains 1105 probes for mature human miRNAs and 1121 probes for pre-miRNAs of these miRNAs. Profiling of these non-coding RNAs was completed in three endometrioid endometrial cancers and five control endometria. The PCA of the results obtained from Affymetrix GeneChip miRNA 2.0 array analyzed by histopathology confirmed that cancer tissues clustered separately from control tissues (Fig. 1A). Unsupervised hierarchical clustering analysis was performed using miRNAs differentially expressed between cancer and control tissues. Pathological samples clustered together and separated from control tissues (Fig. 1B). When the endometrial cancer samples were compared with the endometrial control samples, 199 mature miRNAs were found to be differentially expressed (*P < 0.05; 99 up-regulated and 100 down-regulated; Supplementary data, Table S1).

**Quantification of miRNAs related to angiogenesis by qRT–PCR**

The *in silico* study of the target genes for those miRNAs differentially expressed enabled us to select eight miRNAs related to angiogenesis that exhibited a significant differential expression (*P < 0.05) in endometrial cancer in comparison with the control (miR-17-5p, -20a, -200b, -210, -214*, -221, -222 and -424). Additionally, we selected four miRNAs that did not show significant differential expression between endometrial cancer and control tissues but that are known to be related to angiogenesis and cancer (miR-15b, -16, -21 and -125a).

The evidence for the effects of the 12 selected miRNAs on VEGF-A and TSP-1 is summarized in Supplementary data, Table S2.

All the selected miRNAs were quantified by qRT–PCR in 41 endometroid endometrial cancer and in 56 control endometrium samples: eight miRNAs (miR-15b, -17-5p, -20a, -125a, -214*, -221, -222 and -424) were significantly down-regulated in endometrial cancer in comparison with the control endometrium (Fig. 2). Two miRNAs (miR-200b and -210) were significantly up-regulated, while two miRNAs (miR-16 and -21) did not show any significant difference (Fig. 2).

**Angiogenic components in endometrioid endometrial cancer in comparison with control endometrium**

A significant increase in the VEGF-A protein (1039 ± 251 versus 86 ± 8 pg/mg, *P = 0.001) and mRNA levels (19.0 ± 2.9 versus 5.8 ± 0.6, *P < 0.001) was observed in the endometrial cancer group compared with that in the control group (Fig. 3A and B). When the ratio between VEGF-A protein and mRNA was evaluated (Fig. 3C), a higher increase in the VEGF-A protein than in the corresponding mRNA levels was observed in endometrial cancer tissue compared with control endometrium (VEGF-A ratio, *P = 0.016).

Tissue extracts of endometrial cancer displayed a significant increase in the TSP-1 protein levels (440 ± 92 versus 68 ± 9 ng/mg, *P < 0.001) and mRNA levels (3.5 ± 0.7 versus 1.9 ± 0.2 ng/mg, *P < 0.05) in comparison with those in the control (Fig. 3A and B). The TSP-1 protein/mRNA ratio in endometrial cancer was also significantly higher than that in the control endometrium (*P < 0.05, Fig. 3C).

**Correlation between miRNAs and angiogenic components in tissue extracts**

In all the cancer and control samples, significant inverse correlations between VEGF-A protein levels and miR-20a (*r = -0.212, *P = 0.044), miR-125a (*r = -0.237, *P = 0.025), miR-214* (*r = -0.279, **P < 0.016) and miR-424 (*r = -0.237, ***P < 0.001; n = 41 (cancer) and n = 56 (controls)).

![Figure 2](https://academic.oup.com/humrep/article-abstract/27/10/3036/751396/751396) miRNA levels in endometrial cancer and control endometrium. miRNA expression relative to RNU6B was calculated and the results were normalized so that the average relative expression in the controls became 1. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001; n = 41 (cancer) and n = 56 (controls).
microRNA, angiogenesis and endometrial cancer

There was a significant increase in the VEGF-A protein/mRNA ratio (P < 0.05) and a decrease in the miR-214* (P < 0.005), miR-221 (P < 0.05) and miR-222 (P < 0.05), in endometrial cancer extracts of patients with tumor invasion in more than half of the endometrium (stage IB) in comparison with those with superficial infiltration (stage IA; Fig. 4). An increase in the VEGF-A ratio was observed in endometrial cancer extracts from patients with stage III versus cancer extracts from patients with stage I (6.12 ± 3.15 versus 1.88 ± 0.36, P < 0.05).

Furthermore, a significant increase in the VEGF-A ratio was observed in endometrial cancer extracts from tumors of Grades 2 and 3 in comparison with those from differentiated tumors (Grade 1; 3.57 ± 0.77 versus 1.86 ± 0.34, P < 0.05).

miRNAs and angiogenic components of tissue extracts from endometrial cancer by tumor stage and histological tumor grade

In the present study, we identified 199 miRNAs in a miRNA expression profile that had statistically significant differences between cancer and control endometrial tissues. PCA and hierarchical clusters showed two populations clustered separately. We also validated the expression of 12 miRNAs related to angiogenesis by qRT–PCR. Endometrial cancer was associated with significantly higher VEGF-A expression as well as with lower expression of eight miRNAs (miR-15b, -17-5p, -20a, -125a, -214*, -221, -222 and -424) than the control endometrium. In contrast, two miRNAs (miR-200b and -210) were significantly up-regulated in endometrial cancer. Moreover, significant inverse correlations between VEGF-A protein levels and miR-20a, -125a, -214*, -221, -222 and -424 were observed in endometrial cancer.

miRNAs, which have been implicated in most cellular processes and many diseases, have recently emerged as important regulators of gene expression (Ng et al., 2009). An increasing number of studies have been reported on the relationship between miRNAs and angiogenesis (Suárez et al., 2008; Urbich et al., 2008; Fish and Srivastava, 2009; Wu et al., 2009a; Gotte et al., 2010).

Significant evidence exists implicating the altered expression of several genes as hallmarks of establishment and progression of endometrial cancer, endometriosis and dysfunctional uterine bleeding (Pan and Chegini, 2008). These include ovarian steroid receptors, oncogenic and tumor suppressor genes, proinflammatory and immune-related genes and proteolytic and angiogenic factors. We have previously reported that several miRNAs related to angiogenesis and angiogenic factors are altered in ectopic endometrium compared with the paired eutopic endometrium of women with endometriosis (Ramón et al., 2011).

Several studies have linked high protein levels of VEGF-A to poor prognosis in endometrial cancer (Talvensaari-Mattila et al., 2005; Kamat et al., 2007).

**Discussion**

In the present study, we identified 199 miRNAs in a miRNA expression profile that had statistically significant differences between cancer and control endometrial tissues. PCA and hierarchical clusters showed two populations clustered separately. We also validated the expression of 12 miRNAs related to angiogenesis by qRT–PCR. Endometrial cancer was associated with significantly higher VEGF-A expression as well as with lower expression of eight miRNAs (miR-15b, -17-5p, -20a, -125a, -214*, -221, -222 and -424) than the control endometrium. In contrast, two miRNAs (miR-200b and -210) were significantly up-regulated in endometrial cancer. Moreover, significant inverse correlations between VEGF-A protein levels and miR-20a, -125a, -214*, -221, -222 and -424 were observed in endometrial cancer.

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Several studies have linked high protein levels of VEGF-A to poor prognosis in endometrial cancer (Talvensaari-Mattila et al., 2005; Kamat et al., 2007).
Recently, the dysregulation of miRNA expression in endometrial adenocarcinoma has been reported by several authors (Boren et al., 2008; Chung et al., 2009; Wu et al., 2009b; Cohn et al., 2010; Hiroki et al., 2010; Ratner et al., 2010; Devor et al., 2011; Snowdon et al., 2011). These reports used hybridization array and/or RT–PCR-based methods in order to evaluate miRNA expression in endometrial cancer tissues.

miRNA implication in angiogenesis is bidirectional, so it is possible to classify the miRNAs implicated in angiogenesis regulation under two categories: (i) miRNAs that target genes involved in angiogenesis and (ii) miRNAs whose expression can be modulated by proangiogenic or antiangiogenic stimuli (Caporali and Emanueli, 2011).

We observed an increase in miR-200b and -210 in endometrial cancer tissues than in the control endometrium. Moreover, a positive correlation was obtained between these two miRNAs and the VEGF-A protein level.

The miR-200b is a member of the miR-200 family. This family includes five members located in two genomic clusters: miR-200a, -b and -429 in chromosome 1 and miR-200c and -141 in chromosome 12 (Korpal and Kang, 2008; Lee et al., 2011). The most remarkable similarity among the studies is the up-regulation of the miR-200 family components in endometrial cancer tissues compared with that in normal controls (Chung et al., 2009; Wu et al., 2009a; Cohn et al., 2010; Hiroki et al., 2010; Ratner et al., 2010; Devor et al., 2011; Snowdon et al., 2011). The miR-200 family has been implicated in the epithelial-to-mesenchymal transition, tumor invasion and metastases growth (Gregory et al., 2008).

Although a positive correlation was observed between miR-200b and VEGF-A expression, miR-200c (a member of the miR-200 family) was recently confirmed to bind to the VEGF-A 3’UTR and to repress VEGF-A expression in Ishikawa cells from human endometrial adenocarcinoma (Panda et al., 2012). Moreover, Choi et al. (2011) have confirmed that miR-200b represses VEGF-A expression. Hence, the positive correlation, rather than negative correlations, of miR-200b and VEGF-A observed in the present study could indicate that also the other factors than miRNAs are involved in the regulation of angiogenesis in endometrial cancer.

A significant overexpression of miR–210 in endometrial cancer has been reported previously (Boren et al., 2008; Chung et al., 2009; Wu et al., 2009b; Hiroki et al., 2010; Devor et al., 2011; Snowdon et al., 2011), which has been confirmed by our findings. Moreover, it has been reported that the up-regulation of miR-210 by VEGF-A in ex vivo cultures (Alaiti et al., 2012) and overexpression of miR-210 in HUVEC cells enhances VEGF expression and promotes angiogenesis on Matrigel in vitro (Liu et al., 2012).

Although increases in miR-16 and -21 levels have been reported in ovarian cancer (Iorio et al., 2007; Nam et al., 2008), no differences in these two miRNAs have been reported in endometrial cancer. We evaluated miR-16 because it is a strong VEGF regulator (Karaa et al., 2009) and miR-21 because it induces tumor angiogenesis, thereby increasing VEGF expression (Liu et al., 2011). We found no significant differences in miR-16 and -21 between endometrial cancer and control tissues.

However, we did observe a decrease in miR-15b, -17-5p, -20a, -125a, -214*, -221, -222 and -424 in endometrial cancer in comparison with that in the control endometrium. We also observed an inverse correlation between six of these miRNAs (miR-20a, -125a, -214*, -221, -222 and -424) and the VEGF-A protein levels.
miR-20a and miR-424 are potential miRNAs related to angiogenesis that inhibit angiogenesis by targeting VEGF-A, and they play an important role in regulating angiogenic activity (Wang and Olson, 2009; Chamorro-Jorganes et al., 2011). The down-regulation of these two miRNAs and the increase in VEGF-A levels observed in endometrial cancer tissues in the present report is consistent with that reported in previous studies but it is in contrast with Suarez et al. (2008) who reported that VEGF-A induced miR-20a expression.

The role of the miR-17-92 cluster in angiogenesis is complex (Caporali and Emanueli, 2011). This miR-17-92 cluster encodes six mature miRNAs (miR-17, -18a, -19a, -19b, -20a and -92a; Mendell, 2008; Doebele et al., 2010). We chose to evaluate two of these six: miR-17-5p and miR-20a. The miR-17-5p displays proangiogenic activity (Dews et al., 2006; Suárez et al., 2008), but according to Hua et al. (2006) both miR-17-5p and miR-20a convey antiangiogenic activity by targeting VEGF-A. Recently, Doebele et al. (2010) noted cell-intrinsic antiangiogenic activity of miR-17 and miR-20a in endothelial cells. Here, we report a significant inverse correlation between VEGF-A protein and miR-20a but not miR-17-5p, suggesting that miR-20a displays antiangiogenic activity.

miR-221 and -222 are miRNAs related to angiogenesis with a demonstrated antiangiogenic activity against c-kit (Poliseno et al., 2006). They have been shown to inhibit endothelial cell migration, proliferation and angiogenesis in vitro (Poliseno et al., 2006; Li et al., 2009; Wu et al., 2009a). On the other hand, silencing of miR-214 increased the secretion of pro-angiogenic growth factors, including VEGF-A, whereas miR-214 overexpression had the opposite effect (van Mil et al., 2012). In agreement with previous reports (Boren et al., 2008; Cohn et al., 2010; Hiroki et al., 2010; Snowdon et al., 2011), we also found a decrease in miR-214*, -221 and -424 in the endometrial cancer tissues compared with that in the normal endometrium.

Although no previous studies have indicated a decrease in miR-125a-5p in endometrial cancer, recent studies have shown that the expression of miR-125a-5p is down-regulated in other human cancers such as gastric, breast and ovarian cancers which is consistent with our results (Nam et al., 2008; Guo et al., 2009; O’Day and Lal, 2010; Nishida et al., 2011).

As previously reported (Kamat et al., 2007), an increase in VEGF-A protein levels has been observed in endometrial cancer. Moreover, an increase in TSP-1 protein levels has been also observed. However, the increase in proangiogenic VEGF-A is higher than the increase in antiangiogenic TSP-1 expression (Fig. 3) and indicating a net increase in angiogenesis. Furthermore, although TSP-1 is an antiangiogenic factor, Seki et al. (2001) have described a positive correlation of TSP-1 with angiogenesis in endometrial carcinoma. Thus, the role of TSP-1 in relation to angiogenesis is not clearly delimited.

The increased VEGF-A ratio (protein/mRNA levels) in endometrioid endometrial cancer suggests a differential regulation by one or several posttranscriptional mechanisms involved in the final protein production. One of these mechanisms is the regulation by miRNAs, which bind to target mRNAs mediating translational repression.

The decrease in several miRNAs implicated in angiogenesis in endometrioid endometrial cancer, as well as the negative correlation of miR-20a, -125a, -214*, -221, -222 and -424 with VEGF-A protein levels, suggests that some of these miRNAs could be, directly or indirectly, responsible for the increased VEGF-A ratio observed in endometrial cancer.

Our results point to miRNAs as partially responsible molecules for the angiogenic disequilibrium observed in endometrial adenocarcinoma. The different expression of miRNAs could modulate the expression of angiogenic and antiangiogenic factors, which may play an important role in the pathogenesis of endometrial cancer.

In addition, our finding that tumors with deep infiltration in the myometrium show a higher increase in the protein/mRNA VEGF-A ratio and lower miR-214*, -221 and -222 levels opens new insights toward the establishment of a biomarker profile and the development of new therapeutic strategies for tumors with poor prognosis.

However, the validation of target genes of these differentially expressed miRNAs is necessary to estimate the role of miRNA in the post-transcriptional regulation of angiogenic factors in endometrial cancer. Future functional studies in cell culture models are needed to confirm the VEGF-A/TSP-1 targeting by the miRNAs found in this paper. The findings of the present study have potential implications for diagnostics and therapeutics of endometrial carcinoma.

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

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Authors’ roles
L.A.R. and A.B.-B. participated in study design, performed analyses and interpretation of data and wrote the manuscript. J.G. participated in study design, has provided patients of the study and has contributed analysis and interpretation of data and critical revision. M.C. participated in study execution, analysis of data and critical revision. F.E. has contributed to study design, interpretation of data and critical revision. A.E. developed the idea for the paper, formulated the study design, participated in analysis and interpretation of data and wrote the manuscript. J.G.-E. formulated the study design, has provided patients of the study and has contributed analysis and interpretation of data and critical revision and wrote the manuscript. All authors have approved the final version of the manuscript.

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Conflict of interest

None declared.

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