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MicroRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors

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STUDY QUESTION: Could an aberrant microRNA (miRNA) expression profile be responsible for the changes in the angiogenic and fibrinolytic states observed in endometriotic lesions?

SUMMARY ANSWER: This study revealed characteristic miRNA expression profiles associated with endometriosis in endometrial tissue and endometriotic lesions from the same patient and their correlation with the most important angiogenic and fibrinolytic factors.

WHAT IS ALREADY KNOWN?: An important role for dysregulated miRNA expression in the pathogenesis of endometriosis is well documented. However, to the best of our knowledge, there are no reports of the relationship between angiogenic and fibrinolytic factors and miRNAs when endometrial tissue and different types of endometriotic lesions from the same patient are compared.

STUDY DESIGN, SIZE, DURATION: Case–control study that involved 51 women with endometriosis and 32 women without the disease (controls).

PARTICIPANTS/MATERIALS, SETTING, METHODS: The miRNA expression profiles were determined using the GeneChip miRNA 2.0 Affymetrix array platform, and the results were analysed using Partek Genomic Suite software. To validate the obtained results, 12 miRNAs differentially expressed were quantified by using miRCURY LNA™ Universal RT microRNA PCR. Levels of vascular endothelial growth factor (VEGF-A), thrombospondin-1 (TSP-1), urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) proteins were quantified by ELISA.

MAIN RESULTS AND THE ROLE OF CHANCE: Patient endometrial tissue showed significantly lower levels of miR-202-3p, miR-424-5p, miR-449b-3p and miR-556-3p, and higher levels of VEGF-A and uPA than healthy (control) endometrium. However, tissue affected by ovarian endometrioma showed significantly lower expression of miR-449b-3p than endometrium from both controls and patients, and higher levels of PAI-1 and the angiogenic inhibitor TSP-1. A significant inverse correlation between miR-424-5p and VEGF-A protein levels was observed in patient endometrium, and an inverse correlation between miR-449b-3p and TSP-1 protein levels was observed in ovarian endometrioma. Peritoneal implants had significantly higher levels of VEGF-A than ovarian endometrioma samples.

LIMITATIONS, REASONS FOR CAUTION: Functional studies are needed to confirm the specific targets of the miRNAs differently expressed.

WIDER IMPLICATIONS OF THE FINDINGS: Differences in miRNA levels could modulate the expression of VEGF-A and TSP-1, which may play an important role in the pathogenesis of endometriosis. The higher angiogenic and proteolytic activities observed in eutopic endometrium from patients might facilitate the implantation of endometrial cells at ectopic sites.

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Key words: microRNA / endometriosis / angiogenesis / VEGF-A / fibrinolysis

Introduction

Endometriosis, which is characterized by the presence of endometrial tissue (including glands and stroma) in extra-uterine locations, is one of the most common benign gynaecological diseases (Balasch *et al.*, 1996; Giudice and Kao, 2004; Burney and Giudice, 2012). Like tumour metastases, endometriotic implants require neovascularization if they are to proliferate, invade the extracellular matrix and establish an endometriotic lesion (Laschke and Menger, 2007; Hapangama *et al.*, 2012; McKinnon *et al.*, 2012). There are three clinically distinct endometriotic lesions, which are defined according to their site. Whereas peritoneal implants occur on the peritoneum, endometriomas are cystic lesions sited in the ovary surrounded by endometrioid glands and rectovaginal nodules comprise fibrotic and endometriotic tissue that grow in the rectovaginal space (Gilabert-Estelles *et al.*, 2012; Young *et al.*, 2013). Although it is not clear, whether these endometriotic lesions are variants of the same disease or are different entities (Nisolle and Donnez, 1997; Bulun, 2009), the observed clinical differences require an individualized study of different biological behaviours of each type of lesion.

Although endometriosis is a widely studied disease with a significant impact on the quality of life of patients, the complete pathogenic mechanism remains unresolved. It is believed to be a multifactorial and polygenic disease that involves angiogenesis and proteolysis (Shifren *et al.*, 1996; Donnez *et al.*, 1998; McLaren, 2000; Tan *et al.*, 2002; Gilabert-Estellés *et al.*, 2006, 2007, 2012; Cosín *et al.*, 2009). Our group has reported increased levels of angiogenic and proteolytic factors in endometrium from patients with endometriosis (Gilabert-Estellés *et al.*, 2003, 2005, 2007; Ramón *et al.*, 2005; Cosín *et al.*, 2009, 2010), and indicated that this situation might facilitate the invasive potential of endometrial cells.

Emerging data suggest that dysregulation of microRNA (miRNA) expression may be implicated in the development of endometriosis (Pan *et al.*, 2007, Pan and Chegini, 2008; Toloubeydokhti *et al.*, 2008; Burney *et al.*, 2009; Guo, 2009; Ohlsson-Teague *et al.*, 2009, 2010; Hawkins *et al.*, 2011; Gilabert-Estellés *et al.*, 2012; Braza-Boïls *et al.*, 2013; Laudanski *et al.*, 2013). The apparent abilities of miRNAs to potently regulate the changes in gene expression responsible for endometriosis raise the prospect of using miRNAs as biomarkers and/or therapeutic tools for the disease.

miRNAs are small non-coding RNAs that bind to target mRNAs through translational repression and/or mRNA degradation (Bartel, 2004, 2009). Functional analysis of miRNAs has indicated their regulatory influence on the expression of target genes involved in both physiological and pathological conditions (Ambros, 2004; Burney *et al.*, 2009; Ohlsson-Teague *et al.*, 2009, 2010; Kuokkanen *et al.*, 2010).

Abnormal miRNA expression has been described in different pathological conditions, such as malignancies, cardiovascular diseases, inflammatory disorders and gynaecological pathologies (Urbich *et al.*, 2008; Sonkoly and Pivarcsi, 2009; Zorio *et al.*, 2009; Qin *et al.*, 2012; Ramón *et al.*, 2012). Some reports have described the miRNA expression profiles in eutopic endometrium and ovarian endometrioma from women with endometriosis (Pan *et al.*, 2007; Pan and Chegini, 2008; Toloubeydokhti *et al.*, 2008; Ohlsson-Teague *et al.*, 2009, 2010; Filigheddu *et al.*, 2010). Nonetheless, these reports studied only a few tissues, and neither angiogenic nor proteolytic factors were detected in different tissues from the same patient.

We previously analysed eight miRNAs related to angiogenesis (angio-miRs) and compared the expression of several angiogenic factors in 41 paired samples of eutopic and ovarian endometrioma tissues to 31 endometrial tissue samples from control women (Ramón *et al.*, 2011). The expression of these selected angio-miRs differed between eutopic endometrium samples and ovarian endometrioma samples. This might influence the expression of angiogenic factors and play a role in the pathogenesis of endometriosis.

The objective of the present study was to analyse the miRNA expression profile in endometriosis and to correlate this profile with several angiogenic and fibrinolytic factors in different endometriotic lesions (ovarian endometrioma, peritoneal lesions and rectovaginal nodules) from the same patient with endometriosis compared with control endometrium. To our knowledge, this is the first study that evaluates the miRNA expression profile in different ectopic lesions and eutopic endometrium from the same patient, and compared these with the miRNA profile of endometrium from control women.

Materials and Methods

Clinical groups

Fifty-one Caucasian women with endometriosis were studied (mean age: 34.0 years, range: 20–45). All women underwent laparoscopic surgical examination of the abdominal cavity and complete excision of endometriotic tissue. The presence of the disease was suspected either by clinically or by ultrasonography and confirmed by surgical findings and post-operative pathological examination. Laparoscopic examination of the abdominal cavity excluded the presence of any other pelvic pathology that could potentially confound the data observed. The main symptom for surgery in this group of patients was abdominal pain (74.5%) and sterility (25.5%).

Thirty-two Caucasian asymptomatic women without endometriosis, who underwent surgery for laparoscopic tubal sterilization, were included in the control group (mean age: 36.4 years, range: 27–45). Absence of the disease was confirmed after surgical examination of the abdominal cavity. Meticulous examination of the peritoneum, ovaries, intestine and diaphragm was performed to detect any typical or atypical endometriotic lesions. Biopsies of suspicious areas for endometriosis were confirmed to be negative in these women.

The menstrual phase was identified according to the day of the reproductive cycle and histological analysis of the endometrium. Whereas 26 (51%) women with endometriosis were in the proliferative phase, 25 (49%) were in the secretory phase of the menstrual cycle. Whereas 15 (47%) controls were in the proliferative phase, 17 (53%) were in the secretory phase of the menstrual cycle. Women in the menstrual phase were excluded from the study.

Patients with irregular menstruation or women who had been pregnant or breastfeeding in the previous 6 months were excluded from the study. None of the women had received hormonal treatment for at least 3 months before the study. Informed consent was obtained from all patients and controls, and the study was approved by the Institutional Review Board.

Tissue extracts

Paired ovarian endometriomas and endometrial biopsies (eutopic endometrium) were obtained from 51 patients with endometriosis. Moreover, peritoneal implants were obtained from 18 of the 51 patients, and rectovaginal nodules were obtained from 20 of the 51 patients with endometriosis. Tissue samples from ovarian endometriomas were macroscopically separated from ovarian tissue and peritoneal implants were excised surgically, avoiding the use of electrocautery in the dissection. Rectovaginal nodules were detected through clinical examination, and infiltration of the rectum was discarded by magnetic resonance imaging or rectal ultrasonography. Specimens were obtained after laparoscopic excision of the rectovaginal nodule, and macroscopic tissue sampling was performed at the core of the lesion. Small lesions (measuring <2 cm) or fragmented lesions were rejected to avoid artefacts caused by manipulation of the tissue during surgery or electrocoagulation. Endometrial biopsies from patients (eutopic endometrium) were performed using an atraumatic endometrial suction cannula. Thirty-two endometrial biopsies from women without endometriosis (control endometrium) were performed using the same procedure.

All samples were rinsed in phosphate-buffered saline, and endometriotic tissues were also evaluated microscopically to confirm the diagnosis. Tissue samples were stored in liquid nitrogen until they were processed.

RNA extraction and quality determination

Total RNA was extracted from endometrial and endometriotic tissues and control endometrial tissues using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA), according to manufacturer's protocol. The RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) in all samples. Following manufacturer's specifications, only samples with A260/A280 ratio of $\sim$2.0 and A260/230 ratio in the range of 1.9–2.2 were considered for inclusion in the study. RNA integrity was analysed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). We established a RNA integrity number cut-off value of $\geq$7.0 for microarray assays; this was based on the definition used by Ibberson *et al.* (2009) to define good RNA quality.

Analysis of miRNA expression profiles

For each sample, 500 ng of total RNA was labelled with the FlashTag Biotin RNA Labelling Kit (Genisphere, Hatfield, PA, USA) according to manufacturer's recommendations. Briefly, a tailing reaction was carried out at 37°C for 15 min (10 μl of total RNA and spike control oligos mix, 1× reaction buffer, 2.5 mM $MnCl_2$, 1.33 μM ATP and 1 μl Poly A polymerase enzyme), and this was followed by 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 terminate the reaction.

Labelled RNAs were hybridized on Affymetrix GeneChip miRNA 2.0 arrays (Affymetrix, Santa Clara, CA, USA) at 48°C and 60 rpm for 16 h in the presence of total biotin-labelled RNA, hybridization mix, formamide, DMSO, eukaryotic hybridization controls and control oligonucleotide B2. Immediately following hybridization, the arrays were washed and stained with streptavidin–phycoerythrin conjugate in the GeneChip® Fluidics Station 450. Finally, they were scanned using a GeneChip® Scanner 3000 7G.

Microarray data analysis

For data analysis, Affymetrix *.cel data* files were imported into PARTEK Genomic Suite software (PARTEK, St. Louis, MO, USA) and normalized using the Robust Multi-array Analysis (RMA) algorithm. Following the miRNA expression workflow, normalization of data included RMA, background correction, quartile normalization, log2 transformation values and median polish according to the Genisphere indications for the FlashTag Biotin labelling kit. After ANOVA statistical analysis, the miRNA generated lists were used for further analysis including only miRNAs with a *P*-value of <0.05.

Principal component analysis (PCA) was performed in all array data from hybridized samples, so they were reviewed according to their characteristic miRNA expression profiles. Hierarchical clustering representation of differentially expressed miRNA from all studied samples allowed to identify samples with similar of miRNA expression patterns, according to *P*-values and fold-change criteria. Compared with PCA supervised hierarchical clustering represents, only the generated lists of miRNAs that are differentially expressed.

Identification of miRNA target genes by computational tools

The bioinformatic tools mirbase.org, microrna.org, targetscan.org and Diana tools were used to predict the genes targeted by miRNAs expressed differently in different categories: ovarian endometrioma, eutopic endometrium and control endometrium.

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

Differentially expressed miRNAs with targets implicated in angiogenesis, proteolysis or endometriosis selected in microarray studies were tested by real-time quantitative RT-PCR (qRT-PCR) in a larger cohort of samples, including the samples in which microarray experiments were performed (32 control endometrial samples, 51 paired samples of eutopic endometria and ovarian endometrioma, 18 peritoneal implants and 20 rectovaginal nodules of the 51 patients).

The study involved 12 miRNAs (miR-16-5p, -29c-3p, -138-5p, 202-3p, -373-3p, -411-5p, -411-3p, -424-5p, -449b-3p, -556-3p, -636, -935) and the small nucleolar RNA RNU6B, which provided an endogenous control, which was detected stably expressed in quantitative real-time RT–PCR runs.

Quantification was performed by miRCURY LNA™ Universal RT microRNA PCR (Exiqon, USA). This method is based on a universal reverse transcription followed by a real-time PCR amplification with LNA™ enhanced primers. The protocol was performed as outlined in the instruction manual, employing a Light cycler 480 instrument (Roche).

Total protein extraction and quantification

Cytosolic and membrane protein extracts from endometriotic and endometrial tissues were obtained as previously described (Bouchet-Bernet *et al.*, 1996). Total protein concentration for all the tissue extracts was determined using the BCA protein assay (Pierce, Rockford, IL, USA). Fraction-V bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was used for calibration. Samples and standards were analysed in duplicate.

Vascular endothelial growth factor, thrombospondin-1, urokinase plasminogen activator and plasminogen activator inhibitor-1 protein quantification in tissue lysates

Vascular endothelial growth factor (VEGF-A) protein levels were 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 both human VEGF-A_{165} and VEGF-A_{121} isoforms. The intra-assay and inter-assay variabilities were 4–6 and 7–10%, respectively. The lower detection limit was 15 pg/ml.

Thrombospondin-1 (TSP-1) protein levels were 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-assay and inter-assay variabilities were 5–6 and 8–11%, respectively. The lower detection limit was 1.56 ng/ml.

Urokinase plasminogen activator (uPA) protein levels were quantified by a commercially available ELISA (Zymutest uPA, Hyphen Biomed, France), which measures single-chain urokinase (scuPA) and the high-molecular-weight form of uPA (HMW-uPA) with similar efficiency. The intra-assay and inter-assay variation coefficients were 3–5 and 8–11%, respectively. The lower detection limit was 0.12 ng/ml.

Plasminogen activator inhibitor-1 (PAI-1) protein levels were quantified by a commercially available ELISA (Imubind tissue PAI-1, America Diagnostica, USA). The assay detects free and complexed PAI-1 and is insensitive to PAI-2. The intra-assay and inter-assay variation coefficients were 3–4 and 6–8%, respectively. The lower detection limit was ~0.25 ng/ml.

Statistical analysis

All variables were checked for normal distribution by using the Kolmogorov–Smirnov test. Differences in the studied variables between two groups were analysed using the unpaired Student *t*-test or Mann–Whitney U test. Differences in the studied variables among several groups were analysed by a one-way ANOVA test or Kruskal–Wallis test.

miRNA data quantified by real-time qRT-PCR are presented as fold changes relative to the women without endometriosis group (control endometrium = 1). Values are expressed as mean ± SEM.

Levels of significance in correlations between variables were calculated by the bivariate Pearson correlation test. *P*-values of <0.05 (two-tailed) were considered significant. All these tests were performed using the statistical package SPSS Release 20 for Windows (SPSS Inc.).

Results

miRNA expression profiles determined by microarray analysis

The GeneChip miRNA 2.0 Array contains 1105 probes for mature human miRNAs and 1105 probes for pre-miRNAs of these miRNAs (miRBase version 15). Profiling of these non-coding RNAs was completed in seven eutopic endometrial tissues and three ovarian endometrioma tissues from patients, and from endometrial tissues from five healthy controls. Results generated from the three categories using the Affymetrix GeneChip 2.0 were analysed by PCA using Partek Genomic Suite Software. This graphical algorithm showed a relatively close relationship between the expression profiles of control and eutopic endometrium, which were both relatively isolated from ovarian endometrioma (Fig. 1A). When the three sample categories were compared, we found 156 mature miRNAs that were differentially expressed at least 1.3-fold ($P < 0.05$; 79 up-regulated and 77 down-regulated) in ovarian endometrioma or in eutopic endometrium or in both tissues compared with healthy tissue (Supplementary data, Table SI). Supervised hierarchical clustering of differentially expressed miRNAs showed closer expression signatures in control and eutopic endometrium, with ovarian endometrioma clustering separately from control and eutopic endometrium (Fig. 1B).

We next performed an '*in silico*' study of the genes predicted to be targeted by the 156 differentially expressed miRNAs to investigate which of them might regulate the expression of the most important factors involved in angiogenesis and fibrinolysis, or that had been implicated in endometriosis. This analysis led us to select 12 miRNAs for qRT-PCR validation that employed miRCURY LNA Universal RT microRNA PCR to

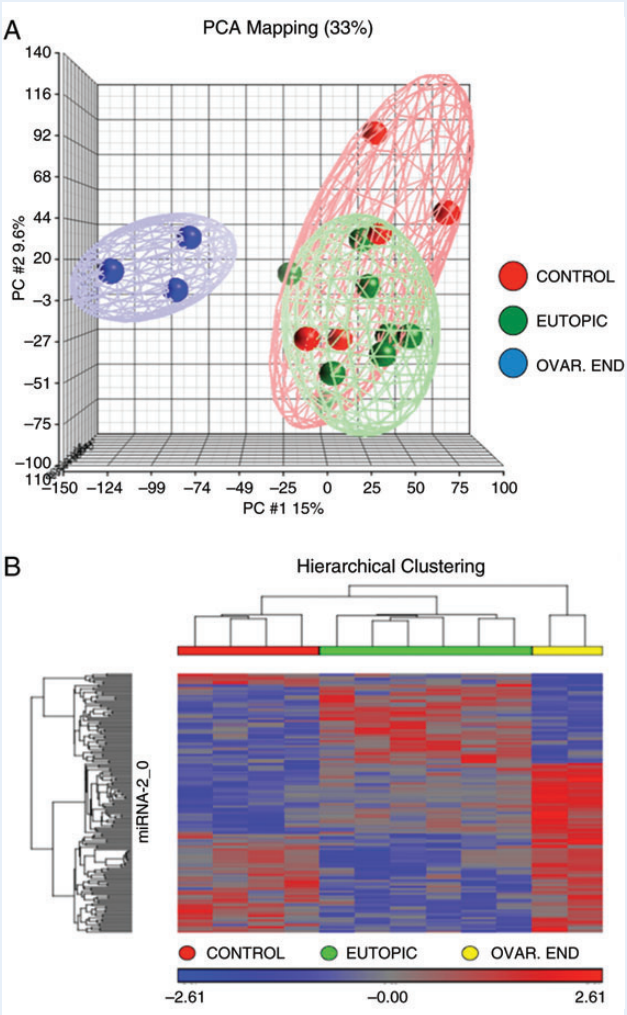

Figure 1 Graphic algorithms showing miRNA expression profiles from endometrial tissues and ovarian endometrioma from patients and control endometrium. (**A**) Principal component analysis applied to the expression of all probes on the Affymetrix GeneChip miRNA 2.0 array. (**B**) Hierarchical cluster analysis of differentially expressed miRNAs from eutopic endometrium, ovarian endometrioma and control endometrial samples.

Table I **miRNA microarray expression and targets of miRNA selected for the PCR experiments.**

miRNA (v. 15)[a]	miRNA (v. 20)[b]	miRNA sequence 5′–3′	Endometrioma versus control		Eutopic versus control		Target
			Fold-change	*P*-value	Fold-change	*P*-value	
miR-16	miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	−1.06806	0.00073	−1.00307	0.77231	VEGFA, EGFR2, BCL2, FGFR1, COX2
miR-29c	miR-29c-3p	UAGCACCAUUUGAAAUCGGUUA	1.52029	0.00588	1.19676	0.09986	VEGFA, PDGFB-C, THSD4 (TSP-1D4), SERBP1(PAI-1 mRNA binding protein), ADAMTS2, 5–7, 9, 17–19
miR-138	miR-138-5p	AGCUGGUGUUGUGAAUCAGGCCG	1.14975	0.00012	1.49604	0.02856	ADAMTS5, BCL2, TNFF4
miR-202	miR-202-3p	AGAGGUAUAGGGCAUGGGAA	6.16021	0.00339	1.11787	0.49639	THBS1 (TSP-1), GLI1[c], IL6R, MMP1, FGF5, FGF11, IL6, IL10
miR-373*	miR-373-3p	GAAGUGCUUCGAUUUUGGGGUGU	−1.59872	0.01366	−1.5995	0.01087	VEGFA, IL8, ADAMST18, MMP24, TIMP3,ESR1
miR-411	miR-411-5p	UAGUAGACCGUAUAGCGUACG	1.30273	0.01779	1.0831	0.20828	CDH2, ADAMST19
miR-411*	miR-411-3p	UAUGUAACACGGUCCACUAACC	2.41509	0.01738	1.73618	0.01988	ADAMTS1, HIF1α, CDH2
miR-424	miR-424-5p	CAGCAGCAAUUCAUGUUUUGAA	1.13317	0.01904	1.22434	0.17864	VEGFA, IL1, FGF2
miR-449b*	miR-449b-3p	CAGCCACAACUACCCUGCCACU	−2.10914	0.02846	−1.09801	0.40826	MMP-16, IL6R, PDGFRA, PDGFRB
miR-556-3p	miR-556-3p	AUAUUACCAUUAGCUCAUCUUU	−1.37746	0.06692	−1.42387	0.00112	VEGFA, ADAMST1, SERBP1(PAI-1 mRNA binding protein), CDN7
miR-636	miR-636	UGUGCUUGCUCGUCCCGCCCGCA	−1.76706	0.12815	−1.52172	0.04888	ADAMTS14, SERBP1, PDGFRA, FGF12
miR-935	miR-935	CCAGUUACCGCUUCCGCUACCGC	−1.68699	0.84800	−1.4834	0.01543	SERBP1, FGF1

[a]Referred to miRBase database release (version 15).
[b]Referred to miRBase database release (version 20). miRNAs are named in microarray according to miRBase version 16. However, the current classification is referred to miRBase 20 release.
[c]Zhao *et al.* (2013).

examine a larger cohort of samples than used in our initial analysis (Table I). Among the selected miRNAs, six miRNAs (miR-29c-3p, -138, -202-3p, -411-5p, -411-3p, -424-5p) were significantly up-regulated in ovarian endometrioma or in eutopic endometrium compared with the control endometrium, and six miRNAs (miR-16, -373-3p, -449b-3p, -556-3p, -636, -935) were significantly down-regulated in both ovarian endometrioma or eutopic endometrium when compared with their levels in control endometrium.

Quantification of selected miRNAs by real-time qRT-PCR in endometriotic lesions and eutopic endometrial tissues compared with control endometrium

The expression of 12 selected miRNAs was quantified by qRT-PCR in 32 control endometrial samples and 51 paired samples of eutopic endometria and ovarian endometrioma from the same patient. We found that eutopic endometrium expressed significantly less miR-202-3p, miR-424-5p, miR-449b-3p and miR-556-3p than control endometrium (Table II, Fig. 2A–C and E). However, ovarian endometrioma expressed significantly higher levels of miR-29c, -138, -202-3p, -373-3p and -411-5p in comparison with control endometrium (Table II, Fig. 2Dand F–H), whereas miR-449b-3p was significantly less abundant in samples of ovarian endometrioma compared with control endometrium (Table II, Fig. 2B). In the case of miR-202-3p, the up-regulation was near to a 100-fold increase in ovarian endometrioma compared with control endometrium and a 200-fold changes increase compared with with eutopic endometrium (Fig. 2D).

Moreover, these selected miRNAs were quantified in 18 peritoneal and 20 rectovaginal endometriotic lesions from the 51 patients to assess whether or not these miRNAs were dysregulated in these ectopic lesions. Peritoneal implants and rectovaginal lesions showed significantly higher expression of miR-16-5p, -29c-3p, -138-5p, -202-3p, -411-5p, -424-5p and -935 than in control and eutopic endometrium (Table II, Fig. 2A, D and F–H). In contrast, in ovarian endometrioma and rectovaginal lesions, levels of miR-449b-3p were lower than those in control endometrium (Table II, Fig. 2B). Moreover, peritoneal implants as well as eutopic endometrium expressed significantly less miR-556-3p than control endometrium.

miRNAs in endometriotic lesions in comparison to eutopic endometrium

The levels of miR-29c-3p, -138, -411-5p and 424-5p were higher in ovarian endometrioma samples, peritoneal implants and rectovaginal lesions than in eutopic endometrium from patients ($P < 0.001$) (Table II, Fig. 2A, D and F–H). Moreover, levels of miR-202-3p were significantly higher ($P < 0.001$) in ovarian endometrioma, peritoneal implants and rectovaginal lesions than in samples of eutopic endometrium (Table II, Fig. 2D).

Angiogenic and fibrinolytic components in endometriotic lesions and eutopic endometrial tissues compared with control endometrium

Whereas levels of VEGF-A and uPA were significantly higher in eutopic endometrium than in control endometrium (Table III, Fig. 3A and C), no significant differences were observed in TSP-1 levels between eutopic endometrium and control endometrium (Table III, Fig. 3B).

Levels of TSP-1 and PAI-1 were significantly higher ($P < 0.001$) in ovarian endometrioma than in control endometrial tissues (Table III, Fig. 3B and D). Levels of VEGF-A and uPA were not significantly different between ovarian endometrioma and control endometrium (Table III, Fig. 3A and C).

Table II miRNAs levels in tissue extracts from endometrium and endometriotic lesions from women with endometriosis relative to those in endometrium from women without endometriosis (control endometrium).

	Control endometrium, $n = 32$	Eutopic endometrium, $n = 51$	Ovarian endometrioma, $n = 51$	Peritoneal endometriosis, $n = 18$	Rectovaginal endometriosis, $n = 20$
miR-16-5p	1.00 ± 0.08	1.69 ± 0.18	1.72 ± 0.27	6.12 ± 0.70$^{***, \#\#\#, \&\&\&}$	10.70 ± 2.50$^{***, \#\#\#, \&\&\&}$
miR-29c-3p	1.00 ± 0.15	0.96 ± 0.07	7.67 ± 0.79$^{***, \#\#\#}$	10.35 ± 1.43$^{***, \#\#\#}$	17.11 ± 3.16$^{***, \#\#\#, \&\&\&}$
miR-138-5p	1.00 ± 0.19	2.15 ± 0.54*	4.47 ± 0.71$^{***, \#\#\#}$	12.89 ± 2.39$^{***, \#\#\#}$	16.03 ± 2.84$^{***, \#\#\#, \&\&\&}$
miR-202-3p	1.00 ± 0.16	0.56 ± 0.07**	113.6 ± 14.30 $^{***, \#\#\#}$	11.38 ± 5.03$^{*, \#\#\#}$	15.92 ± 6.15$^{*, \#\#\#, \&\&\&}$
miR-373-3p	1.00 ± 0.11	0.89 ± 0.08	1.58 ± 0.16*	2.18 ± 0.42$^{*, \#\#}$	1.59 ± 0.50
miR-411-5p	1.00 ± 0.14	0.90 ± 0.08	3.73 ± 0.38$^{***, \#\#\#}$	4.28 ± 0.67$^{***, \#\#\#, \&\&\&}$	7.53 ± 0.97$^{***, \#\#\#, \&\&, \phi}$
miR-411-3p	1.00 ± 0.08	0.90 ± 0.06	1.23 ± 0.13	1.32 ± 0.29*	2.39 ± 0.43$^{**, \#\#\#, \&\&, \phi}$
miR-424-5p	1.00 ± 0.14	0.71 ± 0.07*	1.07 ± 0.15$^{\#}$	1.76 ± 0.46$^{*, \#\#}$	1.89 ± 0.35$^{*, \#\#\#, \&}$
miR-449b-3p	1.00 ± 0.15	0.71 ± 0.10*	0.28 ± 0.05$^{***, \#\#\#}$	1.07 ± 0.52	0.47 ± 0.10$^{**, \#}$
miR-556-3p	1.00 ± 0.21	0.59 ± 0.09*	0.99 ± 0.17	0.32 ± 0.12$^{**, \&\&}$	1.35 ± 0.53
miR-636	1.00 ± 0.09	1.11 ± 0.11	0.95 ± 0.11	1.76 ± 0.36	1.78 ± 0.59
miR-935	1.00 ± 0.13	0.86 ± 0.008	1.35 ± 0.23	4.95 ± 1.68$^{*, \#\#, \&}$	3.15 ± 0.61$^{***, \#\#\#, \&\&}$

Data are expressed as mean ± SEM. miRNA expression is presented as fold-change relative to women without endometriosis (control endometrium = 1).
Any group versus control endometrium: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Any group versus eutopic endometrium: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.
Any group versus ovarian endometrioma: $^{\&}P < 0.05$; $^{\&\&}P < 0.01$; $^{\&\&\&}P < 0.001$. Rectovaginal versus peritoneal endometriosis: $^{\phi}P < 0.05$.

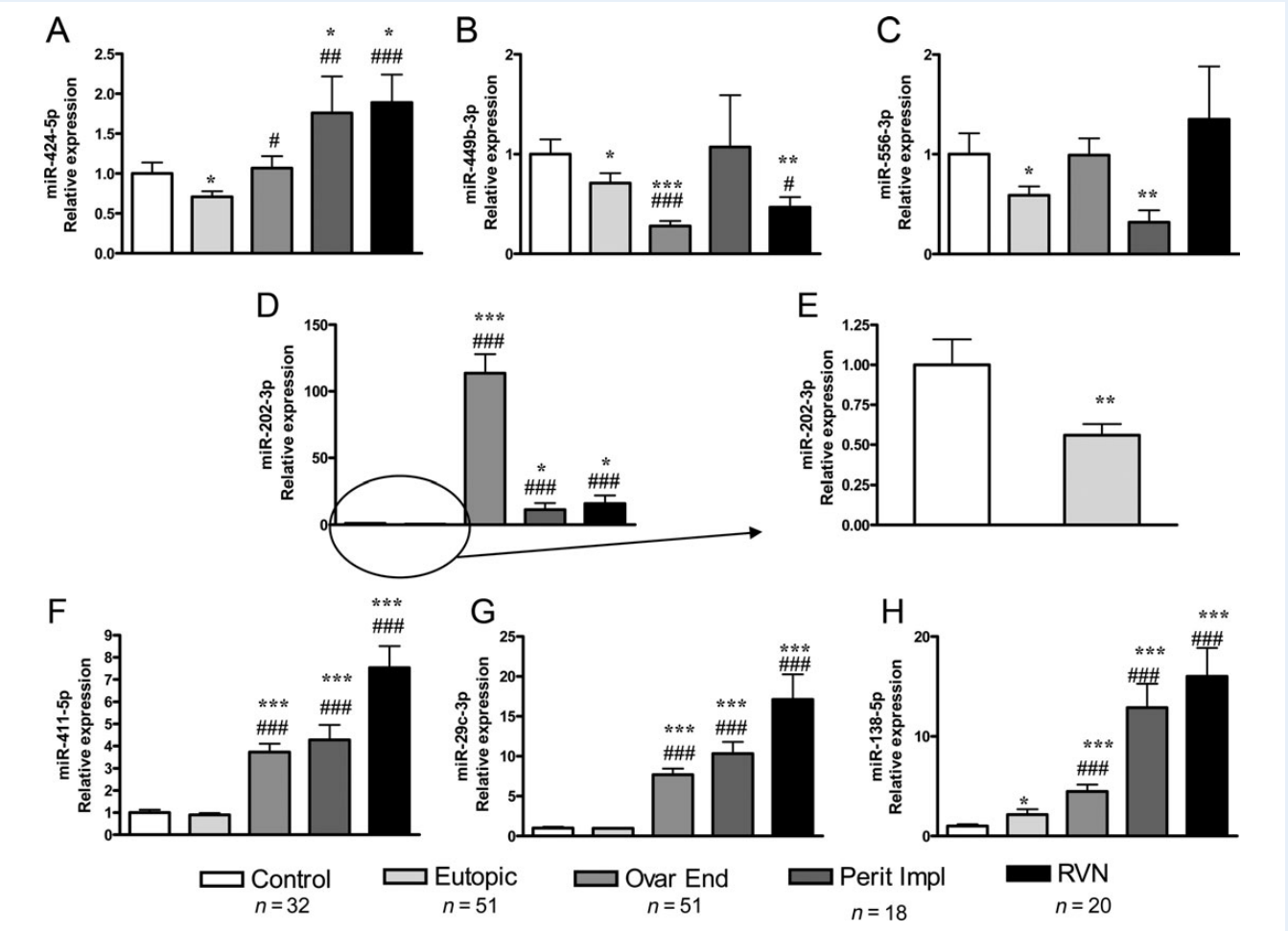

Figure 2 Results from validation by qRT-PCR of miR-424-5p, -449b-3p, -556-3p, -202-3p (down-regulated in eutopic endometrium compared with control endometrium), and miR-202-3p, -411-5p, -29c-3p and -138-5p (up-regulated in endometriotic lesions). (**A**) miR-424-5p. (**B**) miR-449b-3p. (**C**) miR-556-3p. (**D** and **E**) miR-202-3p. (**F**) miR-411-5p. (**G**) miR-29c-3p. (**H**) miR-138-5p. miRNA expression is presented as fold-change relative to control (control endometrium = 1). Data are expressed as mean + SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control endometrium. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus eutopic endometrium. Control: control endometrium ($n = 32$); Eutopic: eutopic endometrium ($n = 51$); Ovar. End: ovarian endometrioma ($n = 51$); Perit. Impl: peritoneal implant ($n = 18$); RVN: rectovaginal nodule ($n = 20$).

Levels of VEGF-A were significantly higher in peritoneal implants than in control endometrium (Table III, Fig. 3A and C). In contrast, rectovaginal lesions showed an increase in TSP-1 ($P < 0.05$) levels and PAI-1 levels ($P < 0.01$) compared with control endometrium (Table III, Fig. 3B and D).

Comparison of miRNAs and protein levels in endometriotic lesions

Peritoneal lesions showed a significant increase in VEGF-A levels and a significant decrease in miR-556-3p compared with the values found in ovarian endometrioma (Tables II and III). Moreover, peritoneal lesions showed a significant decrease in TSP-1 levels in comparison to ovarian endometrioma and rectovaginal lesions (Table III).

In rectovaginal nodules, a significant decrease in TSP-1 was observed in comparison to ovarian endometrioma (Table III). Moreover, rectovaginal lesions showed a significant increase in miR-411-5p and miR-411-3p compared with ovarian endometrioma and peritoneal lesions (Table II).

Peritoneal and rectovaginal lesions also showed a significant increase in miR-138-5p and miR-935 in comparison to ovarian endometrioma (Table II).

Correlation between differentially expressed miRNAs and angiogenic and fibrinolytic components in tissue extracts

Given that miRNAs often inhibit the translation of their target mRNA, we studied the correlation between miRNA and protein levels. We found a significant inverse correlation between miR-424-5p expression and VEGF-A protein levels ($r = -0.380$, $P = 0.006$) in eutopic endometrium from women with endometriosis and a significant inverse correlation between miR-449b-3p and TSP-1 protein levels ($r = -0.314$, $P = 0.02$) in ovarian endometrioma.

Moreover, there was a significant inverse correlation between miR-424-5p and VEGF-A protein levels in control endometrium ($r = -0.352$, $P < 0.05$).

Table III Vascular endothelial growth factor-A , thrombospondin-1 , urokinase and plasminogen activator inhibitor type 1 protein levels in tissue extracts from endometrium and endometriotic lesions from women with endometriosis and endometrium from women without endometriosis (control endometrium).

	Control endometrium, $n = 32$	Eutopic endometrium, $n = 51$	Ovarian endometrioma, $n = 51$	Peritoneal endometriosis, $n = 18$	Rectovaginal endometriosis, $n = 20$
VEGF-A protein (pg/mg)	94 ± 11	218 ± 34**	118 ± 16$^{\#\#}$	445 ± 60***, $^{\#\#\#, \&\&\&}$	316 ± 50**, $^{\#, \&\&}$
TSP-1 protein (ng/mg)	55 ± 8	102 ± 17	941 ± 102***, $^{\#\#\#}$	131 ± 74$^{\&\&\&}$	329 ± 99***, $^{\#, \&\&, \phi}$
uPA protein (ng/mg)	1.08 ± 0.09	1.71 ± 0.21*	1.63 ± 0.23	0.20 ± 0.05$^{\#\#\#, \&\&\&}$	0.31 ± 0.08$^{\#\#, \&\&}$
PAI-1 protein (ng/mg)	2.17 ± 0.30	4.36 ± 0.73*	18.58 ± 2.95***, $^{\#\#\#}$	3.75 ± 1.66*, $^{\&\&\&}$	5.36 ± 1.17***, $^{\&\&}$

Data are expressed as mean ± SEM.
Any group versus control endometrium: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
Any group versus eutopic endometrium: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.
Any group versus ovarian endometrioma: $^{\&}P < 0.05$; $^{\&\&}P < 0.01$; $^{\&\&\&}P < 0.001$.
Rectovaginal versus peritoneal endometriosis: $^{\phi}P < 0.05$.

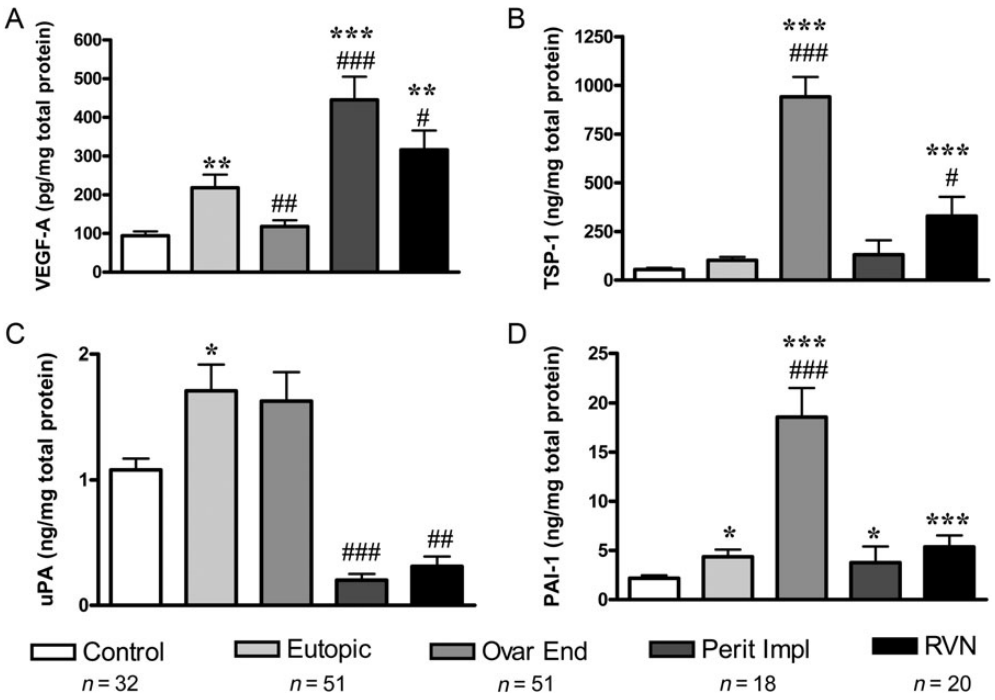

Figure 3 Protein levels of vascular endothelial growth factor-A (VEGF-A), thrombospondin-1 (TSP-1), urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) from different tissues. (**A**) VEGF-A. (**B**) TSP-1. (**C**) uPA. (**D**) PAI-1. Data are expressed as mean + SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control endometrium. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus eutopic endometrium. Control: control endometrium ($n = 32$); Eutopic: eutopic endometrium ($n = 51$); Ovar. End: ovarian endometrioma ($n = 51$); Perit. Impl: peritoneal implant ($n = 18$); RVN: rectovaginal nodule ($n = 20$).

Influence of the phase of menstrual cycle on miRNA levels

To evaluate the possibility that the phase of the menstrual cycle might affect the expression of miRNAs, we performed the statistical analysis on data collected at different phases of the cycle. No significant differences were observed between the secretory and proliferative phases for the different groups for any of the miRNAs studied.

Discussion

In the present study, we identified 156 mature miRNAs (79 up-regulated and 77 down-regulated) that were differentially expressed at least 1.3-fold in ovarian endometrioma and/or eutopic endometrium compared with control endometrial tissues applying microarray technology to obtain miRNA expression profiles. Supervised hierarchical clustering and PCA showed that whereas control and eutopic endometrium had

similar miRNA profiles, ovarian endometrioma showed different expression patterns. The '*in silico*' study of the target genes for those differentially expressed miRNAs enabled us to select 12 miRNAs to validate by qRT-PCR in all the samples. Patient endometrium showed higher VEGF-A levels and lower expression of miR-202-3p and miR-449b-3p compared with control endometrium. In addition, ovarian endometrioma showed significantly higher expression of the angiogenic inhibitor TSP-1 and lower expression of miR-449b-3p than control endometrium. Moreover, miR-29c-3p and miR-202-3p were more abundant in endometriotic tissues than control and patient endometrium.

To our knowledge, this is the first study to evaluate the expression profiles of miRNAs in different ectopic lesions and eutopic endometrium from the same patient, and to compare this with endometrium from control women.

miRNA expression levels of eutopic and ectopic lesions from women with endometriosis have been described recently (Pan *et al.*, 2007; Toloubeydokhti *et al.*, 2008; Burney *et al.*, 2009; Ohlsson-Teague *et al.*, 2009, 2010; Filigheddu *et al.*, 2010; Ramón *et al.*, 2011; Laudanski *et al.*, 2013), but only a few studies have reported differences in miRNA expression in the eutopic endometrium from women with and without endometriosis (Pan *et al.*, 2007; Toloubeydokhti *et al.*, 2008; Burney *et al.*, 2009; Laudanski *et al.*, 2013).

The present study revealed that miR-202-3p expression was significantly lower in eutopic endometrium samples and significantly higher in ovarian endometrioma samples when compared with control endometrium. Recently, it has been reported (Zhao *et al.*, 2013) that miR-202-3p down-regulates the expression of the glioma-associated oncogene homolog 1 (GLI1) transcription factor. This protein regulates the expression of genes involved in proliferation, migration, invasion and angiogenesis through its effects on regulators, such as BCL-2, CD24, metalloproteinase-2 (MMP-2) and MMP-9 (Carpenter and Lo, 2012). GLI1 also regulates the transcription of VEGF-A (Cao *et al.*, 2012; Carpenter and Lo, 2012; Santoni *et al.*, 2013; Zhao *et al.*, 2013). In addition, the anti-apoptotic protein BCL-2 is overexpressed in the eutopic endometrium of women with endometriosis (Burney *et al.*, 2009; Burney and Giudice, 2012). This BCL-2 overexpression could be modulated by miR-202-3p by regulating the expression of the GLI1 transcription factor. Therefore, the significantly reduced levels of miR-202-3p observed in eutopic endometrium compared with control endometrium could contribute to the increased levels of VEGF-A observed in this tissue. In contrast, the increase of miR-202-3p observed in ovarian endometrioma might account, at least in part, for the low angiogenic activity and low-invasive capacity of this tissue. These findings suggest that dysregulation of miRNA during endometriosis might play a pivotal role establishing endometriotic lesions by affecting different physiological processes.

miR-424-5p targets VEGF-A and plays an important role in down-regulating the angiogenic activity of this protein (Wang and Olson, 2009; Chamorro-Jorganes *et al.*, 2011). The lower levels of miR-424-5p and higher levels of VEGF-A protein in eutopic endometrium samples from patients than samples from controls, as well as the inverse correlation between miR-424-5p and VEGF-A protein levels observed in the present study, suggest that this miRNA might account, at least in part, for the higher VEGF-A levels observed in eutopic endometrium from patients compared with controls. In the present report, lower levels of miR-556-3p and higher levels of VEGF-A have been found in eutopic endometrium and peritoneal implant samples from patients when compared with endometrium from controls. Because one of the targets of miR-556-3p is VEGF-A, this miRNA also might contribute for the increase in VEGF-A levels in eutopic endometrium and peritoneal lesions. These increased angiogenic properties of endometrium might be essential to the initial formation of active peritoneal implants and the development of the vascular network that facilitates the growth and invasion of the ectopic tissue.

It has been reported that miR-449b-3p is down-regulated in ectopic endometrium compared with control endometrium (Hawkins *et al.*, 2011). Our results confirm these findings in a larger number of samples from women with and without endometriosis. Others also reported decreased function of several miRNAs, including miR-449b-3p, and overexpression of miR-204 in endometrial cancer cells compared with healthy endometrial cells; these changes repress migration, invasion and extracellular matrix adhesion in endometrial cancer cells (Chung *et al.*, 2012). The reduced level of miR-449b-3p in ovarian endometrioma relative to healthy tissue might account for the low-invasive capability of these ectopic tissues and the frequent clinical finding that preserved normal tissue is located in the vicinity of the endometriotic lesion.

Remodelling of extracellular matrix protein plays a critically important role in the establishment of endometriotic lesions. Abnormal expression of components of metalloproteinase systems at the mRNA level has been reported in both the endometrium and endometriotic tissue of women affected by endometriosis (Ramón *et al.*, 2005; Klemmt *et al.*, 2007). The present study indicated that miR-29c-3p is more abundant in endometriotic tissues (ovarian endometrioma, peritoneal lesions and rectovaginal nodule) than in healthy endometrial tissue. Given that miR-29c-3p targets different extracellular matrix genes, our results support previous studies that suggest a potential role for a network of miRNAs in the remodelling process that leads to implantation of endometrial tissue outside the uterus and to the formation of endometriotic lesions (Ohlsson-Teague *et al.*, 2009, Filigheddu *et al.*, 2010; Hawkins *et al.*, 2011).

miR-138-5p has been related to several biological processes, including proliferation, migration, invasion and the epithelial to mesenchymal transition in various cancers (Liu *et al.*, 2011; Chakrabarti *et al.*, 2013; Qiu *et al.*, 2013; Wang *et al.*, 2013). It has been postulated that, similar to tumoural metastasis, the epithelial to mesenchymal transition may be a key mechanism that underlies the induction of peritoneal invasion in endometriosis (Matsuzaki and Darcha, 2012; Young *et al.*, 2013). In the present report, we found that miR-138-5p is up-regulated in all studied ectopic lesions. Hence, we hypothesized that miR-138-5p regulates a transition in the cellular morphology to a mesenchymal phenotype.

Endometriosis seems to be a progressive disease and a reduction in the angiogenic activity of endometriotic lesions has been observed in advanced stages (Nisolle *et al.*, 1993). Peritoneal lesions showed higher VEGF-A levels and lower miR-556-3p levels than ovarian endometrioma. On the other hand, ovarian endometrioma showed higher TSP-1 levels and lower VEGF-A levels than peritoneal implants. These results suggest that peritoneal endometriotic implants are lesions with a high potential of angiogenesis and invasion of extracellular matrix, while ovarian endometriomas are lesions with low capability of remodelling the surrounding tissue.

There are defined clinical differences between different types of lesions in endometriosis. While rectovaginal nodules are formed of fibrotic tissue that infiltrate deeply in the retroperitoneum or the pelvic viscera, ovarian endometriomas are cystic structures that grow inside the ovary

respecting surrounding ovarian parenchyma. Peritoneal implants are a wide variety of lesions with typical or atypical appearance. Red active peritoneal implants are characterized by neovascularization an adhesion formation. These biological differences might explain a different expression in VEGF-A, TSP-1 and several studied miRNAs depending on the site of endometriosis.

Further functional studies are necessary to confirm the specific targets of the differently expressed miRNAs in endometrial and endometriotic tissues. Nonetheless, our findings point out that these molecules are attractive candidates in the search for novel diagnostic biomarkers to guide therapeutic interventions to treat endometriosis.

Supplementary data

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

Authors' roles

A.B.-B. developed the idea for the paper, participated in study design, performed analyses and interpretation of data and wrote the manuscript. J M.-A performed analyses and interpretation of data. J.G, participated in study design, has provided patients of the study and have contributed analysis and interpretation of data and critical revision. D.S.-I. performed array analysis and interpretation of data. F.E. 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. developed the idea for the paper, formulated the study design, have provided patients of the study and have 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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