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ORIGINAL ARTICLE *Gynaecology*

Peritoneal fluid modifies the microRNA expression profile in endometrial and endometriotic cells from women with endometriosis

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STUDY QUESTION: Could peritoneal fluid (PF) from patients with endometriosis alter the microRNA (miRNA) expression profile in endometrial and endometriotic cells from patients?

SUMMARY ANSWER: PF from patients with endometriosis modifies the miRNA expression profile in endometrial cells from patients.

WHAT IS KNOWN ALREADY: Angiogenesis is a pivotal system in the development of endometriosis, and dysregulated miRNA expression in this disease has been reported. However, to our knowledge, the effect of PF from patients on the miRNA expression profile of patient endometrial cells has not been reported. Moreover, an effect of three miRNAs (miR-16-5p, miR-29c-3p and miR-424-5p) on the regulation of vascular endothelial growth factor (VEGF)-A mRNA translation in endometrial cells from patients with endometriosis has not been demonstrated.

STUDY DESIGN, SIZE, DURATION: Primary cultures of stromal cells from endometrium from 8 control women (control cells) and 11 patients with endometriosis (eutopic cells) and ovarian endometriomas (ectopic cells) were treated with PF from control women (CPF) and patients (EPF) or not treated (0PF) in order to evaluate the effect of PF on miRNA expression in these cells.

PARTICIPANTS/MATERIALS, SETTING, METHODS: MiRNA expression arrays (Affymetrix platform) were prepared from cells (control, eutopic, ectopic) treated with CPF, EPF or 0PF. Results from arrays were validated by quantitative reverse transcription–polymerase chain reaction in cultures from 8 control endometrium, 11 eutopic endometrium and 11 ovarian endometriomas. Functional experiments were performed in primary cell cultures using mimics for miRNAs miR-16-5p, miR-29c-3p and miR-424-5p to assess their effect as VEGF-A expression regulators. To confirm a repressive action of miR-29c-3p through forming miRNA:*VEGFA* duplexes, we performed luciferase expression assays.

MAIN RESULTS AND THE ROLE OF CHANCE: EPF modified the miRNA expression profile in eutopic cells. A total of 267 miRNAs were modified in response to EPF compared with 0PF in eutopic cells. Nine miRNAs (miR-16-5p, miR-21-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p, miR-149-5p, miR-185-5p, miR-195-5p, miR-424-5p) that were differently expressed in response to EPF, and which were potential targets involved in angiogenesis, proteolysis or endometriosis, were validated in further experiments (control = 8, eutopic = 11, ectopic = 11). Except for miR-149-5p, all validated miRNAs showed significantly lower levels (miR-16-5p, miR-106b-5p, miR-130a-5p; miR-195-5p and miR-424-5p, $P < 0.05$; miR-21-5p, miR-29c-3p and miR-185-5p, $P < 0.01$) after EPF treatment in primary cell cultures from eutopic endometrium from patients in comparison with 0PF. Transfection of stromal cells with mimics of miRNAs miR-16-5p, miR-29c-3p and miR-424-5p showed a significant down-regulation of VEGF-A protein expression. However, *VEGFA* mRNA expression after mimic transfection was not significantly modified, indicating the miRNAs inhibited VEGF-A mRNA translation rather than degrading *VEGFA* mRNA. Luciferase experiments also corroborated VEGF-A as a target gene of miR-29c-3p.

LIMITATIONS, REASONS FOR CAUTION: The study was performed in an *in vitro* model of endometriosis using stromal cells. This model is just a representation to try to elucidate the molecular mechanisms involved in the development of endometriosis. Further studies to identify the pathways involved in this miRNA expression modification in response to PF from patients are needed.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first study describing a modified miRNA expression profile in eutopic cells from patients in response to PF from patients. These promising results improve the body of knowledge on endometriosis pathogenesis and could open up new therapeutic strategies for the treatment of endometriosis through the use of miRNAs.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by research grants by ISCIII and FEDER (PI11/00091, PI11/00566, PI14/01309, PI14/00253 and FI12/00012), RIC (RD12/0042/0029 and RD12/0042/0050), IIS La Fe 2011-211, Prometeo 2011/027 and Contrato Sara Borrell CD13/0005. There are no conflicts of interest to declare.

Key words: endometriosis / microRNA / peritoneal fluid / angiogenesis / VEGF-A

Introduction

Endometriosis is one of the most common gynecological diseases, whose prevalence is estimated at ~10% of women of reproductive age and up to 50% of infertile women (Burney and Giudice, 2012). It is characterized by the presence of endometrial tissue outside the uterus, and it is associated with pain and infertility (McKinnon *et al.*, 2012).

Nowadays, the most accepted theory explaining the development of endometriosis is the retrograde menstruation theory (Sampson, 1927). This theory proposes that endometrial fragments migrate to the peritoneum in a retrograde way during menstruation. In women with endometriosis, these endometrial fragments are able to survive, proliferate and develop new vessels to ensure the establishment of ectopic lesions. Although endometriosis is a benign disease, some features are in common with metastatic processes such as an aberrant angiogenesis. Hence, several groups have analyzed the important role of angiogenesis in the pathogenesis of endometriosis (Donnez *et al.*, 1998; Gilabert-Estellés *et al.*, 2007, 2012; Ramón *et al.*, 2011; Rahmioglu *et al.*, 2012; Rocha *et al.*, 2013; Braza-Boïls *et al.*, 2014).

Angiogenesis is a complex process regulated by a balance between promoters (proangiogenic factors) and inhibitors (antiangiogenic factors) and is essential for supplying oxygen and nutrition to tissues. Among these factors, the main regulator for angiogenesis is known to be the vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) system, in which VEGF-A plays a pivotal role (Shibuya, 2008).

Ectopic lesions located in the pelvic peritoneum are immersed in peritoneal fluid (PF); therefore, the study of PF components and their effects on the development of endometriosis is a crucial objective to better understand this pathological condition (Cosín *et al.*, 2010; Berbic and Fraser, 2011; Braza-Boïls *et al.*, 2013; Olkowska-Truchanowicz *et al.*, 2013). In this context, increased levels of peritoneal macrophages and various proinflammatory and proangiogenic cytokines, abnormal T and B lymphocytes and VEGF-A have been reported in the PF from patients (Giudice and Kao, 2004; Gilabert-Estellés *et al.*, 2007; Martínez-Román *et al.*, 1997; Olkowska-Truchanowicz *et al.*, 2013; Rocha *et al.*, 2013). More recently, Berkes *et al.* (2014) described that 49% of studied patients with endometriosis presented neutrophil extracellular traps (NETs) in the PF, whereas control women rarely showed NET formation. They also observed that the highest percentage of NET-positive PFs was observed in patients with Stage I and II disease. These results suggest that NETs could play a role in initiation of the endometriosis.

MicroRNAs (miRNAs) are non-coding RNAs that may regulate angiogenesis through the modulation of RNA translation (Bartel, 2009). Several studies have reported the influence of miRNAs on the expression of proteins involved in physiological and pathological conditions (Burney *et al.*, 2009; Ohlsson-Teague *et al.*, 2009, 2010; Kuokkanen *et al.*, 2010; Ramón *et al.*, 2012). In relation to gynecological diseases, it has been suggested that altered expression of miRNAs may be involved in the development of endometriosis (Pan *et al.*, 2007, 2008; Toloubeydokhti *et al.*, 2008; Burney *et al.*, 2009; Guo, 2009; Ohlsson-Teague *et al.*, 2009, 2010; Hawkins *et al.*, 2011; Ramón *et al.*, 2011; Gilabert-Estellés *et al.*, 2012; Braza-Boïls *et al.*, 2013, 2014; Laudanski *et al.*, 2013). In a recent study (Braza-Boïls *et al.*, 2014), we described dysregulated miRNA expression in endometriosis, including changes in miR-16-5p, miR-29c-3p and miR-424-5p. *In silico* studies showed that these three miRNAs may regulate VEGF-A expression.

Several studies have indicated that miR-424-5p may be involved in angiogenesis regulation (Wang and Olson, 2009; Chamorro-Jorganes *et al.*, 2011).

Moreover, the role of miR-16-5p and miR-424-5p in the cell-intrinsic angiogenic activity of endothelial cells (ECs) has been investigated (Chamorro-Jorganes *et al.*, 2011), and the authors concluded that both miRNAs directly targeted VEGF-A. Therefore, these miRNAs could participate in the regulation of the angiogenic functions of ECs.

In relation to the role of miR-29c in angiogenesis, a previous study in rats confirmed that VEGF-A is a direct target of miR-29a,c specifically suppressing endogenous VEGF-A translation *in vitro* (Yang *et al.*, 2013).

To our knowledge, the direct effect of these three miRNAs (miR-16-5p, miR-29c-3p and miR-424-5p) on the regulation of VEGF-A translation in endometrial and endometriotic cells from patients with endometriosis has not been demonstrated.

In the present study, we investigated the role of PF from patients with endometriosis on the miRNA expression profile in primary cell cultures of stromal cells from control and eutopic endometrium and ovarian endometrioma from patients. Moreover, we evaluated the relationship of miRNAs to the aberrant angiogenesis observed in endometriosis.

Materials and Methods

Ethics statement

Written informed consent was obtained from all patients and controls, and the study was approved by the Ethical Committee from Hospital Universitario y Politécnico La Fe, Valencia, Spain (#2008/0111) and Hospital General Universitario, Valencia, Spain (#PBL00093).

Clinical groups

Patients

Caucasian women with moderate or severe endometriosis (Stages III and IV, revised American Society for Reproductive Medicine classification system, 1997) were studied. All women underwent laparoscopic surgical examination of the abdominal cavity and complete excision of endometriotic tissue. The presence of the disease was suspected either 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.

Controls

Normal endometrial tissues were obtained from fertile women without endometriosis who underwent surgery for tubal sterilization. The absence of endometriosis was confirmed by meticulous examination of the pelvic and extrapelvic peritoneum, ovaries, intestine and diaphragm in order to detect typical or atypical endometriotic lesions.

PF from controls and patients were centrifuged at 1500 × *g* for 30 min at 4°C, filtered through a 0.2 μm pore size membrane, and stored at −80°C.

Women affected by menorrhagia or hypermenorrhea or women who had been pregnant or breastfeeding during the previous 6 months were excluded from the study. None of the women had received any form of hormone therapy for at least 3 months before the study.

Tissue samples and cell lines

In order to isolate stromal cells, 11 endometrial tissues (eutopic cells) (mean age 32 years; range 19–40) and 11 ovarian endometriomas (ectopic cells) from women with moderate or severe endometriosis (Stages III and IV) (mean age 30 years; range 19–42) and control endometrial tissue (control cells) from 8 women without the disease (mean age 36 years; range 24–43) were obtained.

The EC line EA.hy926 was obtained from the American Type Culture Collection (Manassas, VA, USA). ECs were maintained in phenol-red free Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% fetal bovine serum (Life Technologies, Madrid, Spain). A human colon cancer cell line HCT-116 deficient for Dicer (HCT-DK) was a kind gift from Dr Renato Baserga (Thomas Jefferson University, PA, USA). HCT-DK were cultured in McCoy's 5A (Sigma-Aldrich, Madrid, Spain) supplemented with 2 mM glutamine and 10% fetal bovine serum.

PF pools

PF pools consisted of 10 PFs from women with endometriosis (endometriotic PF pool, EPF) (mean age 33 years; range 27–39) and 10 PFs from fertile women without endometriosis (control PF pool, CPF) (mean age 37 years; range 21–47) in the proliferative phase of the menstrual cycle.

Primary cell culture of stromal cells from endometrial and endometriotic tissues and PF exposure

Cell culture and exposure to PF pools were performed as previously described (Braza-Boïls *et al.*, 2013), and functional experiments were performed in cultures at passage 2–4. The cell lines EA.hy926 and HCT-DK were cultured according to the American Type Culture Collection protocols.

Cell transfections

Cells were seeded 24 h before transfection in complete medium without antibiotics and transfected with 100 nM of chemically modified double-stranded RNAs that mimic endogenous miRNAs (miR-16-5p, miR-29c-3p, miR-424-5p or scrambled control) by using the siPORT™ *NeoFX*™ transfection agent from Life Technologies in OPTIMEM according to the manufacturer's instructions. After 24 h, cells were collected for subsequent mRNA and protein analyses. All transfections were performed in triplicate.

RNA extraction

Total RNA from cells stimulated with EPF or CPF and without stimulation (0PF) was extracted using mirVana miRNA isolation kit (Life Technologies), according to the manufacturer's protocol. Total RNA from transfected cell cultures was isolated using Trizol Reagent (Life Technologies). Yield and purity of RNA were measured using a NanoDrop ND-1000 spectrophotometer (Nanodrop Products, Wilmington, DE, USA), and the RNA integrity was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples employed for microarray assays presented a RNA integrity number ≥9.0.

Analysis of miRNA expression profiles

MiRNA expression profiles were studied in primary cell cultures of stromal cells from four eutopic endometrium, three ovarian endometrioma and three control endometrium exposed to EPF, CPF or 0PF. Affymetrix platform, GeneChip miRNA 2.0 arrays (Affymetrix, Santa Clara, CA, USA) were employed according to the manufacturer's protocol. Arrays were prepared in our Array Facility (IIS La Fe, Valencia, Spain). Data analysis was performed employing PARTEK Genomic Suite software (PARTEK, Inc., St Louis, MO, USA) and normalized using the robust multiarray analysis (RMA) algorithm. Analysis of variance (ANOVA) statistical analysis allowed us to generate a list of differently expressed miRNAs, with significance set at a P-value < 0.05.

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

Target genes of differentially expressed miRNAs in response to PF exposure were assessed using the following miRNA binding sites prediction programs: miRBase (http://microrna.sanger.ac.uk/) (Kozomara and Griffiths-Jones, 2011), miRSVR (http://www.microrna.org) (Betel *et al.*, 2010), TargetScan (http://www.targetscan.org) (Lewis *et al.*, 2005) and DIANA-microT (http://diana.imis.athena-innovation.gr) (Paraskevopoulou *et al.*, 2013). Nine miRNAs (miR-16-5p, miR-21-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p, miR-185-5p, miR-195-5p, miR-424-5p) with potential targets involved in angiogenesis, proteolysis or endometriosis were selected to be validated by *quantitative real-time reverse transcription–polymerase chain reaction* (qRT–PCR) in a larger number of experiments, including the cell cultures in which microarray experiments were performed. RNA RNU6B was employed as endogenous control.

Mature miRNAs quantification was performed by miRCURY LNA™ Universal RT microRNA PCR (Exiqon, Vedbaek, Denmark) employing a Light cycler 480 II instrument (Roche Applied Science, Penzberg, Germany).

VEGF-A protein quantification

VEGF-A protein levels from supernatants were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (Human VEGF, IBL International, Hamburg, Germany). No cross-reactivity or interference with platelet-derived growth factor was observed. This assay recognizes human VEGF-A_{165} and VEGF-A_{121} isoforms. The intra-assay and inter-assay variation coefficients were 4–6% and 7–10%, respectively.

VEGF-A protein expression from cells was quantified by western blot (anti-VEGF antibody ab46154, Abcam, Cambridge, UK), which recognizes both human VEGF-A_{165} and VEGF-A_{121} isoforms.

VEGF-A mRNA

VEGF-A mRNA was quantified by qRT–PCR. Briefly, RNA (400 ng) and SuperScript™ III First-Strand Synthesis System (Life Technologies) were used for reverse transcription reactions. *VEGFA* and beta-actin (*ACTB*) (as endogenous reference control) gene expression was quantified by polymerase chain reaction (PCR) (probe references: Hs00900055_m1 and Hs99999903_m1, respectively, from Life Technologies). The $2^{-\Delta Ct}$ method was followed to calculate the relative abundance of mRNA compared with endogenous control expression (Ct = threshold cycle; ΔCt = Ct endogenous control—Ct sample gene).

Luciferase experiments

Plasmid construction

To confirm the repressive action of miR-29c-3p through forming miRNA:-*VEGFA* duplexes, we inserted a fragment of the *VEGFA* 3′ untranslated region (UTR) containing the binding site for this miRNA into a luciferase expression vector, generating the luciferase reporter construct pMIR-VEGFA-3′UTR. Briefly, pMIR-*VEGFA*-3′UTR contained a fragment located at nt +1575-1829 of the *VEGFA* 3′UTR. The PCR fragment was cloned into the pCR 2.1 vector (Life Technologies™). Positive clones were digested with SacI and HindIII (New England Biolabs, Ipswich, MA, USA), and the insert was subcloned into the luciferase reporter plasmid pMIR-REPORT™ (Life Technologies) previously digested with SacI and HindIII. Insertion of the *VEGFA* 3′UTR fragment was checked by sequencing (ABI3130 XL, Life Technologies Corporation, Carlsbad, CA, USA). All sequence analyses and alignments were performed with the SeqmanPro program (Lasergene version 7.1, DNASTAR, Madison, WI, USA).

To generate mutations in the predicted target site for the miR-29c-3p, seven nucleotides located in the seed sequence were deleted using the QuikChange site-directed mutagenesis kit (Agilent Technologies). Sequencing was performed to check for the deletion of the seed sequences. The primers used for cloning and mutagenesis are detailed in Supplementary data, Table SI.

Luciferase vector transfection

MiR-29c-3p mimic was co-transfected with pMIR-*VEGFA*-3′UTR and *Renilla* vector pRL-TK (Promega, Madison, WI, USA) into the HCT-DK cell line. Cells were seeded at a density of 80 000 cells/well in 24-well plates with McCoy's 5A supplemented with 10% fetal calf serum without antibiotics. The following day, cells were co-transfected with scrambled precursor (SCR) or miR-29c-3p mimic (both pMIR-REPORT plasmids—1000 ng/well—wild type or mutated for the miRNA seed site) and 100 ng/well of *Renilla* luciferase control plasmid (pRL-TK, Promega) using Lipofectamine LTX (Life Technologies), according to the manufacturer's instructions. Luciferase assays were performed as previously described (Salloum-Asfar *et al.*, 2014). The enzymatic activities of *Renilla* and firefly luciferases were quantified in a Synergy 2 luminometer (Biotek, Winooski, VT, USA). Each combination of pMIR-REPORT (wild-type and mutated 3′UTR) and pRL-TK was tested in triplicate in five independent experiments. Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well. The normalized data were expressed as changes relative to the data of the cells transfected with 100 nM SCR mimic. SCR was taken as 100%.

Statistical analysis

Results from arrays were analyzed using PARTEK Genomic Suite Software. Comparisons between groups for all other analyses were performed by an unpaired *t*-test. Statistical tests were performed using the Statistical Package for the Social Sciences Release 20 for Windows (SPSS, Inc., Chicago, IL, USA)

Luciferase activity levels were compared between SCR and mimics in wild-type and mutant vectors using linear mixed models. Independent experiments were regarded as a random effects variable in the model, and SCR/miR and WT/MUT factors were regarded as fixed effects. Error bars were used to display SEs, and *P*-values < 0.05 were considered statistically significant. These analyses were performed using R software (version 3.0.2) (r-project.org).

Results

MiRNA expression profiles (Affymetrix platform)

The GeneChip miRNA 2.0 Array contains 1105 human probes for mature miRNAs and 1121 probes for their respective pre-miRNAs.

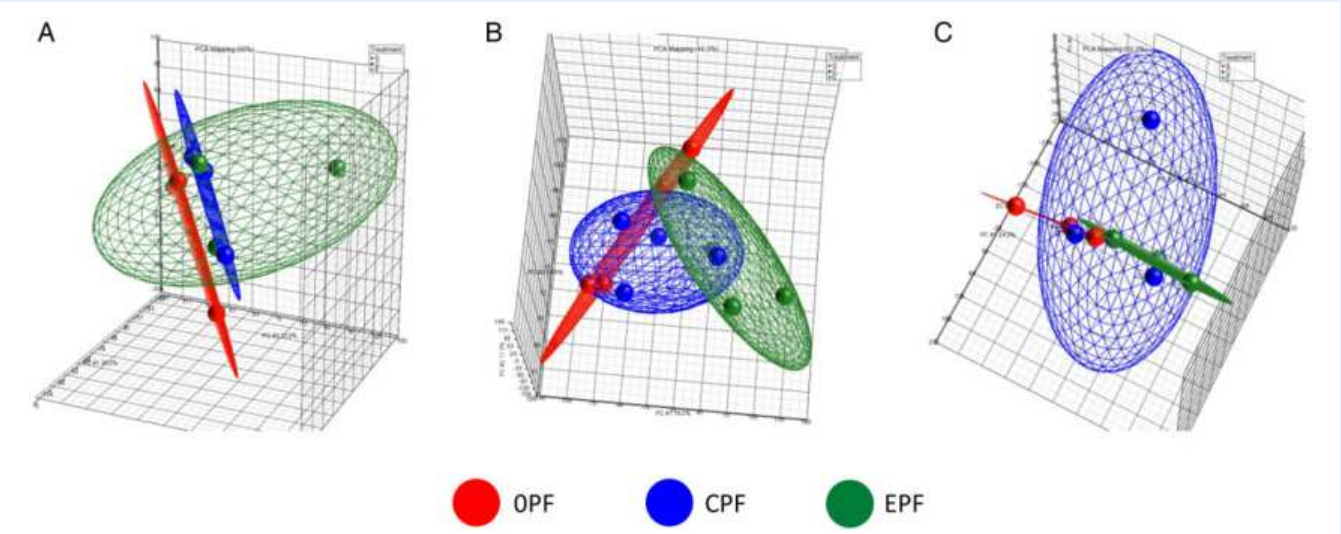

Figure 1 Principal component analysis performed from miRNA expression arrays (Affymetrix): (**A**) control cells, (**B**) eutopic cells and (**C**) ectopic cells. 0PF: without any treatment; CPF: treated with peritoneal fluid from control women and EPF: treated with peritoneal fluid from patients.

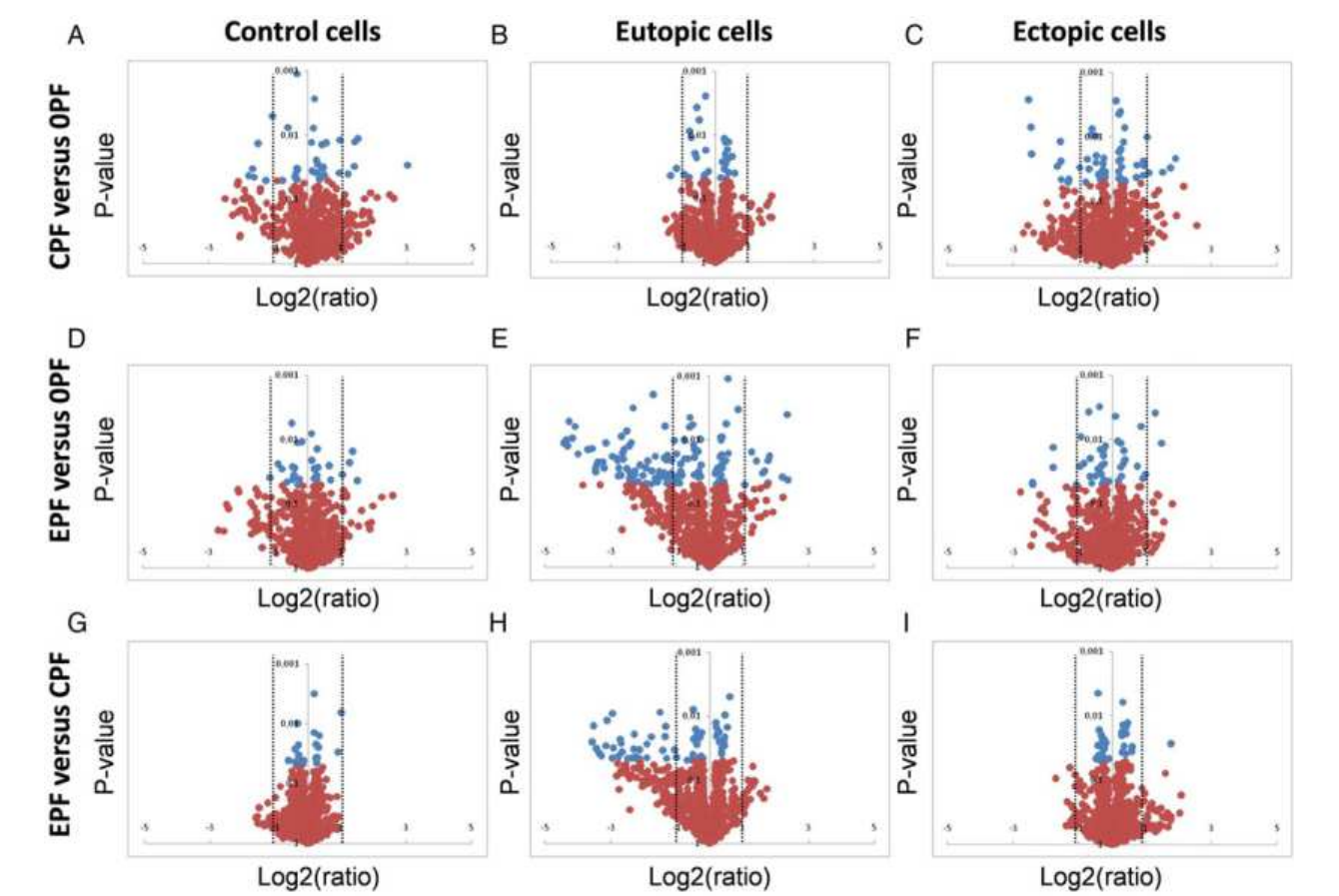

Figure 2 Volcano plots representing miRNA differently expressed in control, eutopic or ectopic cells in response to different treatments.

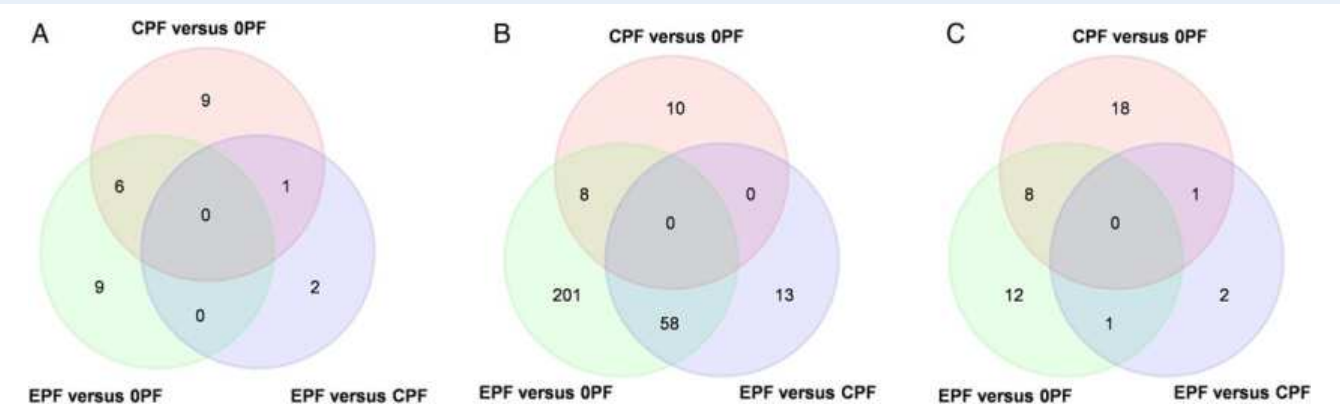

Figure 3 Venn diagrams representing the number of miRNAs dysregulated in each experimental condition: (**A**) control cells, (**B**) eutopic cells and (**C**) ectopic cells. Student's *t*-test.

Profiling of these RNAs was completed for three cultures from control endometrium (control cells), four from endometrium from patients (eutopic cells) and three from ovarian endometriomas (ectopic cells) treated with PFs from patients (EPF), controls (CPF) and without treatment (0PF). Principal component analysis revealed that control cells treated with CPF showed no modification in the miRNA expression pattern in comparison with untreated cells. Nevertheless, the miRNA expression was different in response to EPF (Fig. 1A). In contrast to control cells, eutopic and ectopic cells responded to EPF and CPF in a different way in terms of miRNA expression (Fig. 1B and C). Volcano plots from ANOVA test (Fig. 2) revealed that the major difference in miRNA expression was observed in eutopic cells after EPF treatment

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′	Eutopic cells response to EPF compared with 0PF		Target
			Fold change	*P*-value *	
miR-16	**miR-16–5p**	UAGCAGCACGUAAAUAUUGGCG	−9.96870	0.04321	VEGFA, EGFR2, BCL2, FGFR1, COX2
miR-21	**miR-21-5p**	UAGCUUAUCAGACUGAUGUUGA	−16.96419	0.00620	TIMP3, TGFb2, SERPINB5, VEGFA, BCL2, EGFR, MMP2, HIF1a, MMP8, TGFb, TGFBR1, THBS1, TNFRSF11B
miR-29c	**miR-29c-3p**	UAGCACCAUUUGAAAUCGGUUA	−2.38095	0.01301	VEGFA, PDGFB-C, THSD4 (TSP-1D4), SERBP1, ADAMTS2, 5–7, 9, 17–19
miR-106b	**miR-106b-5p**	UAAAGUGCUGACAGUGCAGAU	−20.34633	0.01002	TGFBR2, MMP2, THSD3, CCNG2, ADAM9, IL8, MMP24, COL4A3, CCND1, TIMP2, CCND2, COL19A, FGF4, VEGFA
miR-130a	**miR-130a-3p**	CAGUGCAAUGUUAAAAGGGCAU	−19.25180	0.00517	SERPINE1, COL4A1, IL6R, COL4A5, VEGFA, COL1A2, SERPINB7, FAS (TNFR superfamily)
miR-149	**miR-149-5p**	UCUGGCUCCGUGUCUUCACUCCC	3.43946	0.04766	GPC1, FGFR1[c], EDNRA, TNFRSF19
miR-185	**miR-185-5p**	UGGAGAGAAAGGCAGUUCCUGA	−19.84907	0.01248	VEGFA, THSD7A, CLDN11, IL17R, HIF3a, EDA2R
miR-195	**miR-195-5p**	UAGCAGCACAGAAAUAUUGGC	−4.99004	0.00318	COL12A1, CDCA4, BCL2L2, VEGFA, CLDN12, CCND1, SERBP1, DICER1, ADAMTS5, GHR, CLDN2, ESRRA, ESRRG, ADAMTS1
miR-424	**miR-424-5p**	CAGCAGCAAUUCAUGUUUUGAA	−2.03838	0.04712	VEGFA, IL1, FGF2

ADAMTS2, 5–7, 9, 17–19: ADAM metallopeptidase with thrombospondin type 1 motif, 2, 5–7, 9, 17–19; BCL2: B-cell lymphoma 2; BCL2L2: BCL2-like 2; CCND1: cyclin D1; CCND2: cyclin D2; CCNG2: cyclin G2; CDCA4: cell division cycle associated 4; CLDN11: claudin 11; CLDN12: claudin 12; COL1A2: collagen, type I, alpha 2; COL4A1: collagen, type IV, alpha 1;COL4A3: collagen, type IV, alpha 3; COL4A5: collagen, type IV, alpha 5; COL12A1: collagen, type XII, alpha 1; COL19A: collagen, type IXX, alpha; COX2: cyclooxygenase 2; DICER1: dicer 1, ribonuclease type III; EDA2R: ectodysplasin A2 receptor; EDNRA: endothelin receptor type A; EGFR2: epidermal growth factor receptor 2; ESRRA: estrogen-related receptor alpha; ESRRG: estrogen-related receptor gamma; FAS (TNFR superfamily): Fas cell surface death receptor; FGF2: fibroblast growth factor 2; FGF4: fibroblast growth factor 4; FGFR1: fibroblast growth factor receptor 1; GHR: growth hormone receptor; GPC1: glypican 1; HIF1a-3a: hipoxia inducible factor 1–3, alpha subunit; IL1: interleukin 1; IL6R: interleukin 6 receptor; IL8: interleukin 8; IL17R: interleukin 17 receptor; MMP2: matrix metalloproteinase-2; MMP8: matrix metalloproteinase-8; MMP24: matrix metalloproteinase-24; PDGFB-C: platelet-derived growth factor polypeptide-C; SERPINE1: plasminogen activator inhibitor type 1; SERPINB5-7: serpin peptidase inhibitor, clade B, member 5–7; TGFb2: transforming growth factor beta 2; TGFBR1: transforming growth factor, beta receptor 1; TGFBR2: transforming growth factor, beta receptor 2; THBS1: thrombospondin 1; THSD3: thrombospondin, type 1, domain containing 3; THSD4: thrombospondin, type 1, domain containing 4; THSD7A: thrombospondin, type I, domain containing 7A; TIMP3-2: tissue inhibitor of metalloproteinases-3-2; TNFRSF11B: tumor necrosis factor receptor superfamily, member 11b; TNFRSF19: tumor necrosis factor receptor superfamily, member 19; VEGFA: vascular endothelial growth factor.

EPF, endometriotic peritoneal fluid; 0PF, without peritoneal fluid.

[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]Chamorro-Jorganes *et al.* (2014).

*ANOVA.

(Fig. 2E and H). Moreover, it should be underlined that the majority of these miRNAs were down-regulated in response to EPF. The comparison between the response to EPF and to 0PF showed that eutopic cells presented the highest number of miRNAs significantly dysregulated: $P < 0.05$ and ± 2-fold change (Fig. 2H). Venn diagrams (Fig. 3) representing all of the differentially expressed ($P < 0.05$) human miRNA probes in the array showed that EPF modified the expression of some miRNAs >12-fold in eutopic cells compared with the other cultures. Among the 267 miRNAs that are modified in response to EPF compared with 0PF in eutopic cells (Fig. 3B), 82 corresponded to mature miRNAs (72 down-regulated and 10 up-regulated) ($P < 0.05$; ± 2-fold change) (Supplementary data, Table SII).

After the *in silico* study of the target genes for those miRNAs differentially expressed in eutopic cells from patients treated with EPF, we selected nine miRNAs related to angiogenesis (miR-16-5p, miR-21-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p, miR-149-5p, miR-185-5p, miR-195-5p, miR-424-5p) for validation by qRT–PCR in a larger number of experiments. Eight of these miRNAs were down-regulated in the expression arrays (miR-16-5p, miR-21-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p, miR-185-5p, miR-195-5p, miR-424-5p), and miR-149-5p was up-regulated. (Table I).

Validation by qRT–PCR

With the exception of miRNA-149-5p, which did not show increased levels, as the arrays results revealed (Fig. 4F), the other eight miRNAs showed statistically significant lower levels after EPF treatment in primary cell cultures from eutopic endometrium from patients (Fig. 4A–E, H and I). MiR-16-5p and miR-424-5p showed lower levels after CPF and EPF treatments in control cells. Ectopic cells reduced the expression of miR-16-5p, miR-29c-3p, miR-106b-5p, miR-130a-5p and miR-185-5p in the presence of both PF pools, but only the change in miR-16-5p was statistically significant.

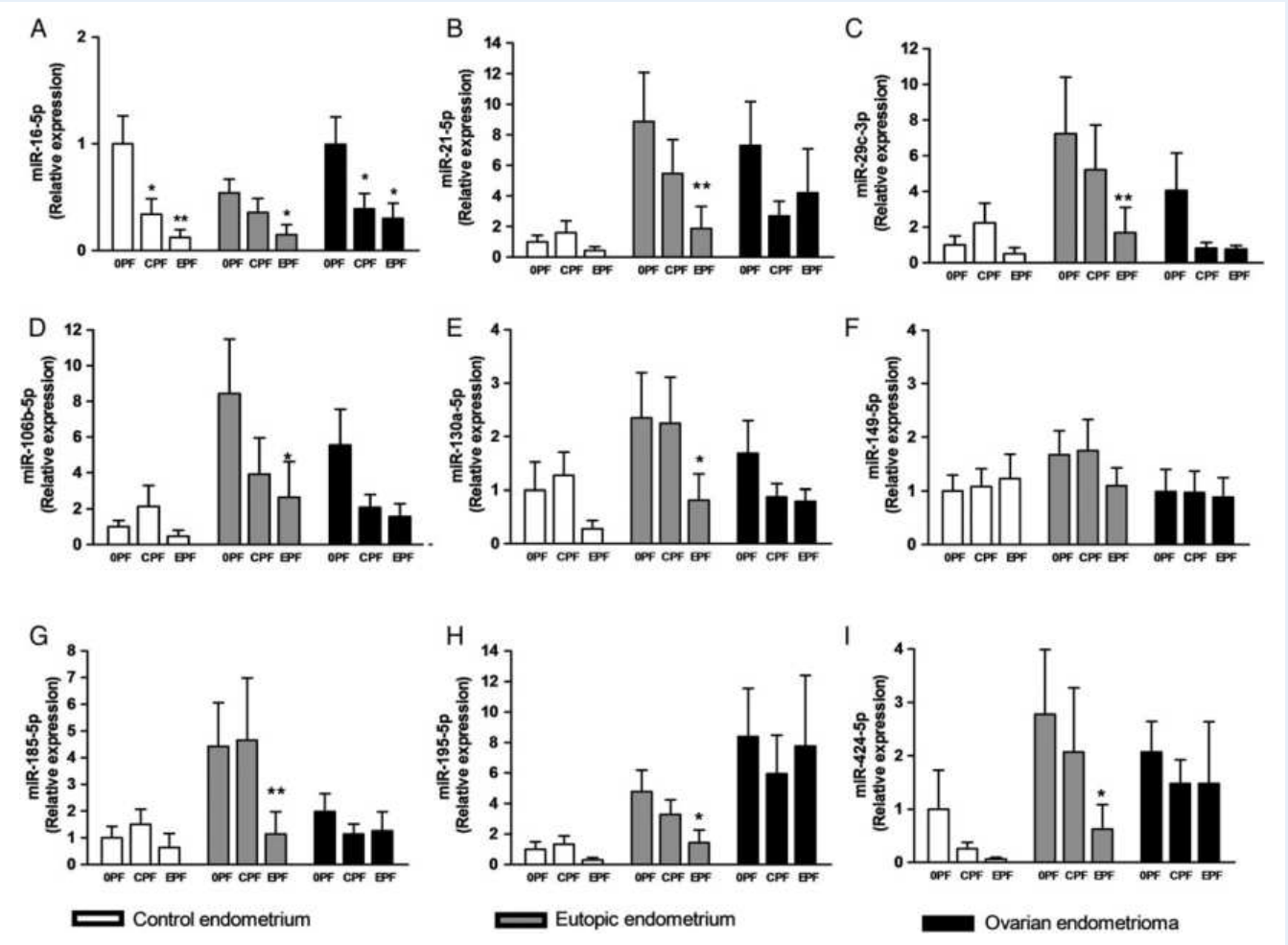

Figure 4 Nine miRNAs were selected from array results in order to be validated by qRT–PCR in control ($n = 8$), eutopic ($n = 11$) and ectopic ($n = 11$) cells treated with: ANOVA test *$P < 0.05$ and **$P < 0.01$.

Functional experiments using mimics

MiR-16-5p, miR-29c-3p and miR-424-5p were significantly down-regulated in eutopic cells after EPF treatment in the array validation phase. Recently, our group published a dysregulated miRNA expression profile in different lesions characteristic of endometriosis, including miR-16-5p, miR-29c-3p and miR-424-5p (Braza-Boïls *et al.*, 2014). Moreover, *in silico* analysis revealed that all three miRNAs could regulate VEGF-A translation (Table I).

To specifically investigate whether VEGF-A expression could be regulated by these three miRNAs, we transfected the EA.hy926 EC line and primary cell cultures from control and patient endometrium with mimics of miR-16-5p, miR-29c-3p and miR-424-5p (Fig. 5).

In order to assess the possible effect of endogenous miRNAs on mimic transfections, miRNAs levels were quantified by qRT–PCR (Supplementary data, Fig. S1). Results validated the effect of exogenous synthetic miRNAs used in the functional studies.

In the EA.hy926 cell line, transfection with miR-16-5p, miR-29c-3p or miR-424-5p mimics induced a reduction in VEGF-A expression versus scrambled mimic of 63 ± 11%, 76 ± 0.9% and 79 ± 0.9% ($P < 0.01$), respectively (Fig. 5A and D). When the same transfections with miR-16-5p, miR-29c-3p or miR-424-5p mimics were performed in primary cell cultures from controls and patient endometrium, VEGF-A expression was reduced versus scrambled mimic by 79 ± 20% ($P = 0.12$), 90 ± 0.2% and 90 ± 0.2% ($P < 0.001$) in endometrial cells from women without the disease (Fig. 5B and D) and 96% ($P < 0.001$), 79% and 78% ($P < 0.01$) in patient endometrial cells, respectively (Fig. 5C and D). In control cell cultures, different doses of mimics (20, 50 and 100 nM) were transfected. *VEGFA* mRNA levels were quantified after transfections, observing no statistically significant modifications in any of the studied cell types (Fig. 5E). Moreover, quantification of VEGF-A protein levels by ELISA showed that mimics seem to act in a dose-dependent manner (Supplementary data, Fig. S2).

Validation of miRNA-VEGF-A interaction

To test the hypothesis that miR-29c-3p can directly modulate VEGF-A expression, *VEGFA* 3′UTR was cloned downstream from the firefly luciferase open reading frame. Either the wild-type reporter construct or the miR-29c-3p binding site deleted construct were co-transfected in different experiments (Fig. 5G) in the HCT116-Dicer KO cell line, with a SCR or a miR-29c-3p mimic.

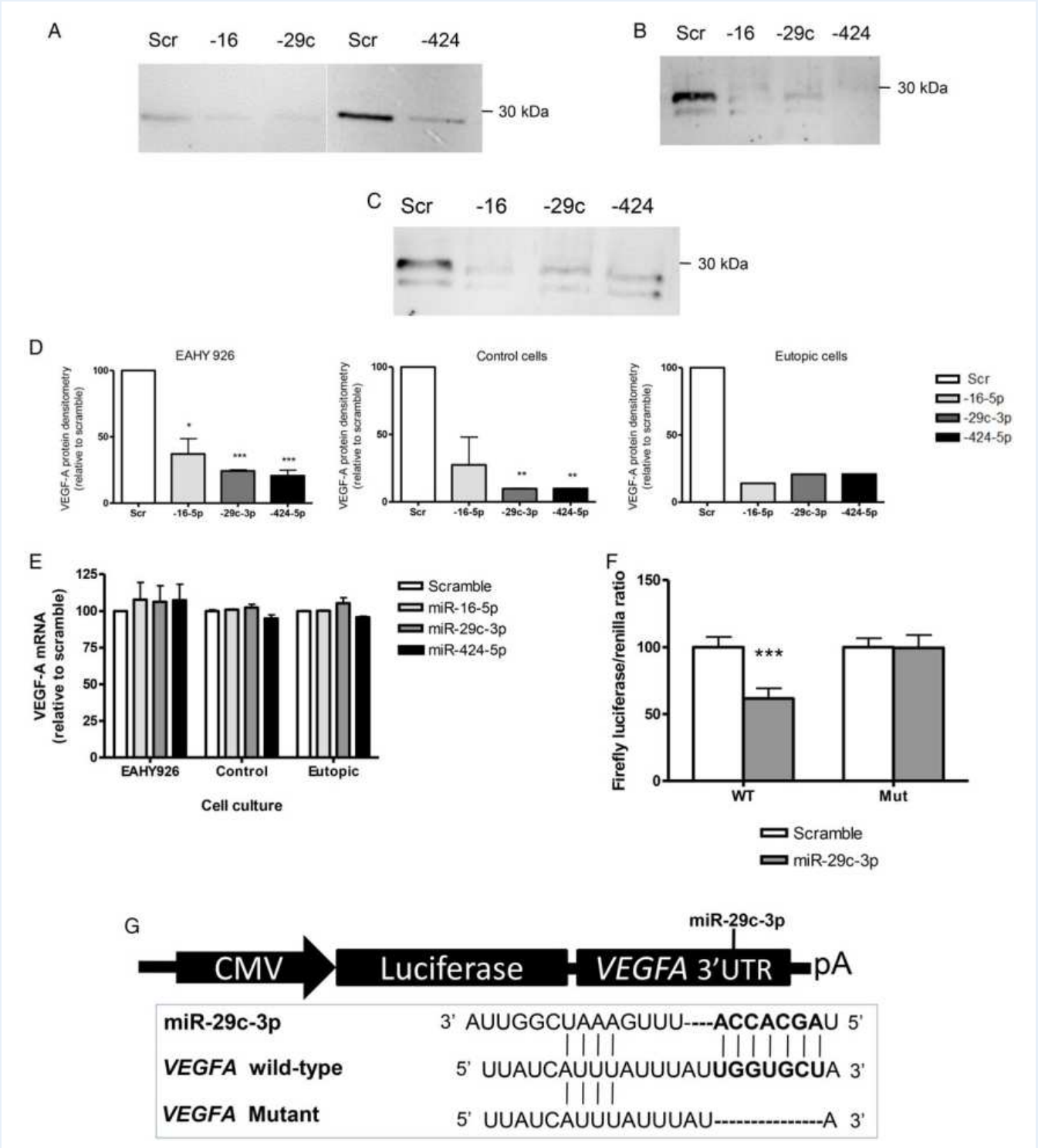

Figure 5 *In vitro* studies. (**A–C**) Representative western blots for VEGF-A after transfection of cells (A: EA.hy 926, B: control cells, C: eutopic cells) with scramble or miR-16-5p, miR-29c-3p and miR-424-5p mimics (100 nM) for 48 h. (**D**) Densitometric analysis of VEGF-A extracellular expression. (**E**) VEGF-A protein levels after control cell transfection with mimics (20, 50 and 100 nM) measured by ELISA. (**F** and **G**) Luciferase assays. (**F**) VEGF-A mRNA levels after mimic transfection (100 nM). (**G**) Schematic representation of miR-29c-3p predicted target site in *VEGFA* 3′ UTR. Complementarities between the seed region (seven nucleotides) of miR-29c-3p and 3′ UTR of *VEGFA* mRNA target site are shown. HCT116—The dicer KO cell line was co-transfected with scramble or miR-29c-3p mimic and pMIR-*VEGFA*-3′ UTR wild-type (WT) or mutated (Mut). All experiments were performed in triplicate ($n = 3$). ANOVA test $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Our results showed that the relative luciferase activity was significantly decreased in cells co-transfected with the wild-type construct and miR-29c-3p (62 ± 8%, $P < 0.001$). However, this inhibition was not observed when co-transfection was performed with the vector containing the specific mutated 3′UTR of *VEGFA* (Fig. 5F), indicating that *VEGFA* 3′UTR could be a direct target of miR-29c-3p.

Discussion

In the present study, we observed that PF from patients modified the miRNA expression profile in endometrial stromal cells from women with endometriosis, including miRNAs involved in angiogenesis. In a previous report (Braza-Boïls *et al.*, 2014), we described a dysregulated miRNA expression profile in endometrial and endometriotic tissues, including miR-16-5p, miR-29c-3p and miR-424-5p, and *in silico* studies showed that these three miRNAs may regulate VEGF-A expression. In the present study, we have performed functional studies employing mimics for these miRNAs, indicating that these miRNAs regulate VEGF-A translation not only in the EA.hy926 cell line but also in cells from endometrial tissues from women with and without endometriosis.

Angiogenesis plays an important role in multiple physiological and pathological processes including gynaecological diseases like endometriosis. Several miRNAs can control the expression of VEGF-A. MiR-29c-3p is a multifunctional miRNA implicated in several processes, including extracellular remodeling and angiogenesis, and can contribute to the formation of endometriotic lesions in patients with endometriosis (Braza-Boïls *et al.*, 2014). A study performed in rats (Yang *et al.*, 2013) demonstrated that VEGF-A is a direct target of miR-29a and miR-29c and these miRNAs suppressed endogenous VEGF-A expression *in vitro*. In the present study, we have observed that the transfection of miR-29c-3p in endometrial and endometriotic cells from patients with endometriosis significantly decreased VEGF-A protein expression. Furthermore, luciferase experiments indicated that VEGF-A is a direct target of miR-29c-3p also in humans.

Both miR-16-5p and miR-424-5p target the same 'seed sequence', the nucleotide sequence in which these miRNAs can bind to *VEGFA* mRNA, which implies that both miRNAs can share most of their target genes. In the present work, we observed a significant reduction in VEGF-A protein expression in primary cell cultures from controls and patients endometrium after transfection with miR-16-5p or miR-424-5p mimics. However, *VEGFA* mRNA expression after mimic transfection was not significantly modified. The decrease in protein levels without significant modification of mRNA levels indicates that these miRNAs mainly inhibit VEGF-A translation without degrading *VEGFA* mRNA, as has been described for several proteins (Braza-Boïls *et al.*, 2013). Indeed, in a previous study (Braza-Boïls *et al.*, 2013), we investigated the influence of PF from women with and without endometriosis on the expression of six miRNAs, including miR-16-5p, that modulate angiogenesis, as well as several angiogenic and proteolytic factors in endometrial and endometriotic cell cultures. We found a significant correlation between the decrease in miR-16-5p and the increase in VEGF-A protein, but not mRNA, in response to PF exposure in endometrial and endometriotic cell cultures.

In a previous report, Chamorro-Jorganes *et al.* (2011) investigated the role of miR-16-5p and miR-424-5p in the angiogenic activity of ECs and showed that both miRNAs directly targeted *VEGFA*. These results are in agreement with results obtained in the present report, in which we have observed that miR-16-5p and miR-424-5p can regulate VEGF-A protein levels in endometrial and endometriotic cells.

In a previous study (Braza-Boïls *et al.*, 2014), we suggested that miR-424-5p contributed, at least in part, to the higher VEGF-A levels observed in the endometrium from patients with endometriosis. Other authors indicated that 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). Moreover, Nakashima *et al.* (2010) reported that down-regulation of mir-424 can contribute to the abnormal angiogenesis in senile hemangioma.

MiRNAs may mediate cell-to-cell communication via exosomes (Boon and Vickers, 2013; Kosaka *et al.*, 2013). However, the mechanisms whereby miRNAs are packaged in exosomes and the selection of miRNAs secreted in each cell state are unclear. Exosomal miRNAs have been characterized in blood, urine and other body fluids, and exosomes can reflect their tissue or cell of origin by the presence of specific surface proteins (Zhang *et al.*, 2015). Moreover, a cell-phenotype modulation induced by miRNAs-enriched exosomes has been described (Hulsmans and Holvoet, 2013; Raposo and Stoorvogel, 2013; Rayner and Hennessy, 2013).

In order to perform miRNA-based communication, three steps are required. In a first step, miRNAs must be secreted from donor cells into exosomes. Second, miRNAs migrate into RNAse-protected vesicles in the recipient cell. And, finally, miRNAs must recognize their mRNA target and repress its translation (Boon and Vickers, 2013).

Although peritoneal macrophages are able to secrete miRNA-rich exosomes (Hulsmans and Holvoet, 2013) and could contribute to the final endometriotic phenotype by means of the aforementioned mechanism, our results showed that PF from patients modified miRNA expression in eutopic cells from patients with endometriosis, indicating not only that peritoneal factors could be involved in the endometriosis pathogenesis, but also that endometrial factors seem to be implicated. However, more studies are required in order to elucidate the mechanisms by which PF from patients is able to modify the receptor cell phenotype.

In conclusion, PF from patients modified the miRNA expression profile in endometrial cells from women with endometriosis. Functional studies employing mimics for miR-16-5p, miR-29c-3p and miR-424-5p suggested that these miRNAs regulate VEGF-A translation not only in EA.hy926 cells but also in primary stromal cells from endometrium from patients with endometriosis and control women. Additional experiments are required in order to elucidate the potential role of miRNA-rich exosomes in this '*in vitro*' model of endometriosis. These promising results really improve the body of endometriosis pathogenesis knowledge that could open up new therapeutic strategies for the treatment of endometriosis through the use of miRNAs.

Supplementary data

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

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Authors' roles

A.B.-B. developed the idea for the paper, formulated the study design, performed analyses and interpretation of data, and manuscript drafting. S.S.-A. performed analyses and interpretation of data. J.M.-A. performed analyses and interpretation of data and wrote the manuscript. B.A. performed analyses and interpretation of data. R.G.-C. participated in analysis and interpretation of data and participated in a critical revision. M.B.-B. performed analyses and interpretation of data. J.G.-O. provided patients for the study. V.V. participated in a 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. provided patients for the study and participated in a critical revision. C.M. developed the idea for the paper, formulated the study design, performed analyses and interpretation of data, and wrote the manuscript. All authors have approved the final version of the manuscript.

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Conflict of interest

None declared.

References

Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;**136**:215–233.

Berbic M, Fraser IS. Regulatory T cells and other leukocytes in the pathogenesis of endometriosis. *J Reprod Immunol* 2011;**88**:149–155.

Berkes E, Oehmke F, Tinneberg HR, Preissner KT, Saffarzadeh M. Association of neutrophil extracellular traps with endometriosis-related chronic inflammation. *Eur J Obstet Gynecol Reprod Biol* 2014;**183**:193–200.

Betel D, Koppal A, Agius P, Sander C, Leslie C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol* 2010;**11**:R90.

Boon RA, Vickers KC. Intercellular transport of microRNAs. *Arterioscler Thromb Vasc Biol* 2013;**33**:186–192.

Braza-Boïls A, Gilabert-Estellés J, Ramón LA, Gilabert J, Marí-Alexandre J, Chirivella M, España F, Estellés A. Peritoneal fluid reduces angiogenesis-related microRNA expression in cell cultures of endometrial and endometriotic tissues from women with endometriosis. *PLoS One* 2013;**8**:e62370.

Braza-Boïls A, Marí-Alexandre J, Gilabert J, Dolors S-I, España F, Estellés A, Gilabert-Estellés J. microRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors. *Hum Reprod* 2014;**29**:978–988.

Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. *Fertil Steril* 2012;**98**:511–519.

Burney RO, Hamilton AE, Aghajanova L, Vo KC, Nezhat CN, Lessey BA, Giudice LC. MicroRNA expression profiling of eutopic secretory endometrium in women with versus without endometriosis. *Mol Hum Reprod* 2009;**15**:625–631.

Chamorro-Jorganes A, Araldi E, Penalva LO, Sandhu D, Fernández-Hernando C, Suárez Y. MicroRNA-16 and microRNA-424 regulate cell-autonomous angiogenic functions in endothelial cells via targeting vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1. *Arterioscler Thromb Vasc Biol* 2011;**31**:2595–2606.

Chamorro-Jorganes A, Araldi E, Rotllan N, Cirera-Salinas D, Suárez Y. Autoregulation of glypican-1 by intronic microRNA-149 fine tunes the angiogenic response to FGF2 in human endothelial cells. *J Cell Sci* 2014;**127**:1169–1178.

Cosín R, Gilabert-Estellés J, Ramón LA, Gómez-Lechón MJ, Gilabert J, Chirivella M, Braza-Boïls A, España F, Estellés A. Influence of peritoneal fluid on the expression of angiogenic and proteolytic factors in cultures of endometrial cells from women with endometriosis. *Hum Reprod* 2010;**25**:398–405.

Donnez J, Smoes P, Gillerot S, Casanas-Roux F, Nisolle M. Vascular endothelial growth factor (VEGF) in endometriosis. *Hum Reprod* 1998;**13**:1686–1690.

Gilabert-Estellés J, Ramón LA, España F, Gilabert J, Vila V, Réganon E, Castelló R, Chirivella M, Estellés A. Expression of angiogenic factors in endometriosis: its relation to fibrinolytic and metalloproteinase (MMP) systems. *Hum Reprod* 2007;**22**:2120–2127.

Gilabert-Estelles J, Braza-Boils A, Ramon LA, Zorio E, Medina P, España F, Estelles A. Role of microRNAs in Gynecological Pathology. *Curr Med Chem* 2012;**19**:2406–2413.

Giudice LC, Kao LC. Endometriosis. *Lancet* 2004;**364**:1789–1799.

Guo SW. Epigenetics of endometriosis. *Mol Hum Reprod* 2009;**15**:587–607.

Hawkins SM, Creighton CJ, Han DY, Zariff A, Anderson ML, Gunaratne PH, Matzuk MM. Functional microRNA involved in endometriosis. *Mol Endocrinol* 2011;**25**:821–832.

Hulsmans M, Holvoet P. MicroRNA-containing microvesicles regulating inflammation in association with atherosclerotic disease. *Cardiovasc Res* 2013;**100**:7–18.

Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, Filippidis C, Dalamagas T, Hatzigeorgiou AG. web server v5.0: service integration into miRNA functional analysis workflows. *Nucleic Acids Res* 2013;**41**:W169–W173.

Kosaka N, Yoshioka Y, Hagiwara K, Tominaga N, Katsuda T, Ochiya T. Trash or treasure: extracellular microRNAs and cell-to-cell communication. *Front Genet* 2013;**4**:173.

Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res* 2011;**39**:D152–D157.

Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N, Pollard JW. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. *Biol Reprod* 2010;**82**:791–801.

Laudanski P, Charkiewicz R, Kuzmicki M, Szamatowicz J, Charkiewicz A, Niklinski J. MicroRNAs expression profiling of eutopic proliferative endometrium in women with ovarian endometriosis. *Reprod Biol Endocrinol* 2013;**11**:78.

Lewis BP, Burge CB, Bartel D. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;**120**:15–20.

Martínez-Román S, Balasch J, Creus M, Fábregues F, Carmona F, Vilella R, Vanrell JA. Transferrin receptor (CD71) expression in peritoneal macrophages from fertile and infertile women with and without endometriosis. *Am J Reprod Immunol* 1997;**38**:413–417.

McKinnon B, Bersinger NA, Wotzkow C, Mueller MD. Endometriosis-associated nerve fibers, peritoneal fluid cytokine concentrations, and pain in endometriotic lesions from different locations. *Fertil Steril* 2012;**97**:373–380.

Nakashima T, Jinnin M, Etoh T, Fukushima S, Masuguchi S, Maruo K, Inoue Y, Ishihara T, Ihn H. Down-regulation of mir-424 contributes to the abnormal angiogenesis via MEK1 and cyclin E1 in senile hemangioma: its implications to therapy. *PLoS One* 2010;**5**:e14334.

Ohlsson Teague E, Van der Hoek K, Van der Hoek M, Perry N, Wagaarachchi P, Robertson S, Print C, Hull L. MicroRNA-regulated pathways associated with endometriosis. *Mol Endocrinol* 2009;**23**:265–275.

Ohlsson-Teague EM, Print CG, Hull ML. The role of microRNAs in endometriosis and associated reproductive conditions. *Hum Reprod Update* 2010;**16**:146–165.

Olkowska-Truchanowicz J, Bocian K, Maksym RB, Białoszewska A, Włodarczyk D, Baranowski W, Ząbek J, Korczak-Kowalska G, Malejczyk J. CD4? CD25? FOXP3? regulatory T cells in peripheral blood and peritoneal fluid of patients with endometriosis. *Hum Reprod* 2013;**28**:119–124.

Pan Q, Chegini N. MicroRNA signature and regulatory functions in the endometrium during normal and disease states. *Semin Reprod Med* 2008; **26**:479–493.

Pan Q, Luo X, Toloubeydokhti T, Chegini N. The expression profile of micro-RNA in endometrium and endometriosis and the influence of ovarian steroids on their expression. *Mol Hum Reprod* 2007;**13**:797–806.

Rahmioglu N, Missmer SA, Montgomery GW, Zondervan KT. Insights into assessing the genetics of endometriosis. *Curr Obstet Gynecol Rep* 2012; **1**:124–137.

Ramón LA, Braza-Boïls A, Gilabert-Estellés J, Gilabert J, España F, Chirivella M, Estellés A. microRNAs expression in endometriosis and their relation to angiogenic factors. *Hum Reprod* 2011;**26**:1082–1090.

Ramón LA, Braza-Boïls A, Gilabert J, España F, Chirivella M, Estellés A, Gilabert-Estellés J. microRNAs related to angiogenesis are dysregulated in endometroid endometrial cancer. *Human Reprod* 2012;**27**: 3036–3045.

Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013;**200**:373–383.

Rayner KJ, Hennessy EJ. Extracellular communication via microRNA: lipid particles have a new message. *J Lipid Res* 2013;**54**:1174–1181.

Rocha AL, Reis FM, Taylor RN. Angiogenesis and endometriosis. *Obstet Gynecol Int* 2013;**2013**:859619.

Salloum-Asfar S, Teruel-Montoya R, Arroyo AB, García-Barberá N, Chaudhry A, Schuetz E, Luengo-Gil G, Vicente V, González-Conejero R, Martínez C. Regulation of coagulation factor XI expression by microRNAs in the human liver. *PLoS One* 2014;**9**:e111713.

Sampson JA. Metastatic or embolic endometriosis, due to the menstrual dissemination of endometrial tissue into the venous circulation. *Am J Pathol* 1927;**3**:93–110.

Shibuya M. Vascular endotelial growth factor-dependent and independent regulation of angiogenesis. *BMB Rep* 2008;**41**:278–286.

Toloubeydokhti T, Pan Q, Luo X, Bukulmez O, Chegini N. The expression and ovarian steroid regulation of endometrial micro-RNAs. *Reprod Sci* 2008;**15**:993–1001.

Wang S, Olson EN. AngiomiRs: key regulators of angiogenesis. *Curr Opin Genet Dev* 2009;**19**:205–211.

Yang L, Engeland CG, Cheng B. Social isolation impairs oral palatal wound healing in Sprague-Dawley rats: a role for miR-29 and miR-203 via VEGF suppression. *PLoS One* 2013;**8**:e72359.

Zhang J, Li S, Li L, Li M, Guo C, Yao J, Mi S. Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics Proteomics Bioinformatics* 2015;**13**:17–24.