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

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Submitted on May 14, 2015; resubmitted on July 17, 2015; accepted on July 30, 2015

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.
**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 development of endometriosis (Cosín et al., 2010; Martínez-Román et al., 2010; Berbic and Dokhti, 2009; Burney et al., 2009; Ramón et al., 2010; Kuokkanen et al., 2010; Roha et al., 2013; Bahra-Boils et al., 2012). In a recent study (Bahra-Boils 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 ultrasound 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 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-Boıls 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 (IS 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), 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-A165 and VEGF-A121 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-A165 and VEGF-A121 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 \( 2^{-\Delta \Delta Ct} \) method was followed to calculate the relative abundance of mRNA compared with endogenous control expression (\( Ct = \) threshold cycle; \( \Delta Ct = Ct \) 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.
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.

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.
cells presented the highest number of miRNAs significantly dysregulated: these miRNAs were down-regulated in response to EPF. The comparison (Fig. 2E and H). Moreover, it should be underlined that the majority of Peritoneal fluid modifies microRNA expression probes in the array showed that EPF modified the expression of some presenting all of the differentially expressed (P < 0.05 and 0PF in eutopic cells (Fig. 3B), 82 corresponded to mature miRNAs (72 down-regulated and 10 up-regulated) (Supplementary data, Table SII). *ANOVA.

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

<table>
<thead>
<tr>
<th>miRNA (v. 15)</th>
<th>miRNA (v. 20)</th>
<th>miRNA sequence 5′–3′</th>
<th>Fold change</th>
<th>P-value *</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-16</td>
<td>miR-16–5p</td>
<td>UAGCGACACGUAAAAUUGCG</td>
<td>−9.96870</td>
<td>0.04321</td>
<td>VEGFA, EGF2R, BCL2, FGFR1, COX2</td>
</tr>
<tr>
<td>miR-21</td>
<td>miR-21–5p</td>
<td>UAGCUCUACACAGUAGUGUA</td>
<td>−16.96419</td>
<td>0.00620</td>
<td>TIMP3, TGFβ2, SERPINB5, VEGFA, BCL2, EGF, MMP2, HIF1α, MMP8, TGFβ2, TGFBR1, THBS1, TNFRSF11B</td>
</tr>
<tr>
<td>miR-29c</td>
<td>miR-29c–3p</td>
<td>UAGCAGCUUUGAAAAUCGUGUA</td>
<td>−2.38095</td>
<td>0.01301</td>
<td>VEGFA, PDGF-B, THSD4 (TSP-1D4), SERBP1, ADAMTS2, 5–7, 19–19</td>
</tr>
<tr>
<td>miR-106b</td>
<td>miR-106b–5p</td>
<td>UAAAGUGCUGACAGUGCAU</td>
<td>−20.34633</td>
<td>0.01002</td>
<td>TGFβ2R, MMP2, THSD3, CCNG2, ADAM9, IL8, MMP24, COL4A3, CCN1, TIMP2, CCND2, COL19A, FGFR, VEGFA</td>
</tr>
<tr>
<td>miR-130a</td>
<td>miR-130a–3p</td>
<td>CAGUGCAAGUUGAAAAAGGCACU</td>
<td>−19.25180</td>
<td>0.00517</td>
<td>SERPINE1, COL4A1, IL6R, COL4A5, VEGFA, COL1A2, SERPINB7, FAS (TNFR superfamily)</td>
</tr>
<tr>
<td>miR-149</td>
<td>miR-149–5p</td>
<td>UCCUGGCGUCCGUCUCUCAUCCCC</td>
<td>3.43946</td>
<td>0.04766</td>
<td>GPC1, FGFR1, EDNRA, TNFRSF19</td>
</tr>
<tr>
<td>miR-185</td>
<td>miR-185–5p</td>
<td>UGGAGAGAAAGGCCAGUCCUGA</td>
<td>−19.84907</td>
<td>0.01248</td>
<td>VEGFA, THSD7A, CLDN11, IL17R, HIF3α, EDAR</td>
</tr>
<tr>
<td>miR-195</td>
<td>miR-195–5p</td>
<td>UAGCAGCAGACAAUUGUUGC</td>
<td>−4.99004</td>
<td>0.00318</td>
<td>COL12A1, CDCA4, BCL2L2, VEGFA, CLDN12, CCND1, SERBP1, Dicer1, ADAMTS3, GHR, CLDN2, ESRR, ESRRG, ADAMTS1</td>
</tr>
<tr>
<td>miR-424</td>
<td>miR-424–5p</td>
<td>CAGCAGCAGUUCUUGUUGAA</td>
<td>−2.03838</td>
<td>0.04712</td>
<td>VEGFA, IL1, FGFR</td>
</tr>
</tbody>
</table>

**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). 1.6-5p 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-29.3p, miR-106b-5p, miR-130a-5p, miR-149.5p, miR-185.5p, miR-195.5p, and miR-424-5p was up-regulated. (Table 2).

**Table II** miRNA microarray expression and targets of miRNA selected for the PCR experiments.
Functional experiments using mimics

MiR-16-5p, miR-29c-3p and miR-424-5p were significantly downregulated 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-Boils 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 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-Boı¨ls 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-Boı¨ls 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-Boı¨ls et al., 2013). Indeed, in a previous study (Braza-Boı¨ls 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-Boı¨ls 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/.

Acknowledgements

The authors thank David Hervás for statistical analysis and Dolors Sánchez-Izquierdo for microarray data analysis.
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. 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.

Funding
This work was supported by ISCIII and FEDER (PI11/00091, PI14/00253, PI14/01309 and FI12/00012), RIC (RD12/0042/0029 and RD12/0042/0050), IIS La Fe 2011-211, Prometeo 2011/027 and 00253, PI14/01309 and FI12/00012), RIC (RD12/0042/0029 and 00253, PI14/01309 and FI12/00012), RIC (RD12/0042/0029 and 00253, PI14/01309 and FI12/00012).

Conflict of interest
None declared.

References


