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Research Article

Differential Expression of MicroRNAs between Eutopic and Ectopic Endometrium in Ovarian Endometriosis

Nicoletta Filigheddu,[1] Ilaria Gregnanin,[1] Paolo E. Porporato,[2] Daniela Surico,[1] Beatrice Perego,[1] Licia Galli,[1] Claudia Patrignani,[2] Andrea Graziani,[2] and Nicola Surico[1]

[1] *Laboratory of Oncological Gynecology, Department of Clinical and Experimental Medicine, and Biotechnology Center for Applied Medical Research, University of Piemonte Orientale "Amedeo Avogadro", 28100 Novara, Italy*
[2] *Laboratory of Biochemistry, Department of Clinical and Experimental Medicine, and Biotechnology Center for Applied Medical Research, University of Piemonte Orientale "Amedeo Avogadro", 28100 Novara, Italy*

Correspondence should be addressed to Nicoletta Filigheddu, nicoletta.filigheddu@med.unipmn.it

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Endometriosis, defined as the presence of endometrial tissue outside the uterus, is a common gynecological disease with poorly understood pathogenesis. MicroRNAs are members of a class of small noncoding RNA molecules that have a critical role in posttranscriptional regulation of gene expression by repression of target mRNAs translation. We assessed differentially expressed microRNAs in ectopic endometrium compared with eutopic endometrium in 3 patients through microarray analysis. We identified 50 microRNAs differentially expressed and the differential expression of five microRNAs was validated by real-time RT-PCR in other 13 patients. We identified *in silico* their predicted targets, several of which match the genes that have been identified to be differentially expressed in ectopic *versus* eutopic endometrium in studies of gene expression. A functional analysis of the predicted targets indicates that several of these are involved in molecular pathways implicated in endometriosis, thus strengthening the hypothesis of the role of microRNAs in this pathology.

1. Introduction

Endometriosis, defined as the growth of endometrial tissue outside the uterine cavity, is a common gynecological disease often resulting in chronic pelvic pain and infertility. The pathogenesis of endometriosis is likely multifactorial and several hypotheses have been suggested to explain the presence of ectopic endometrial tissue and stroma, such as retrograde menstrual reflux [1], immune system defects [2–10], and ectopic presence of endometrial stem cells originating the disease [11]. In addition, there is a growing body of evidence indicating the involvement of genetic factors in the etiology of endometriosis, as it has been calculated that there is a 6–9-fold increased prevalence of this pathology among the 1st-degree relatives of women with endometriosis, compared to the general population [12–18]. Extensive investigations have been performed to characterize the differences between the eutopic and ectopic endometrium in order to better understand and define the molecular basis of the disease and, indeed, several studies have revealed a distinct pattern of gene expression in eutopic and ectopic endometrium [19–24]. The differences in gene expression reported in these works include genes encoding proteins involved in cell adhesion, extracellular matrix remodeling, migration, proliferation, immune system regulation, and inflammatory pathways, thus accounting for the multiple mechanisms hypothesized to be responsible for the establishment of ectopic endometrial implants, including the adhesion of endometrial cells to the pelvic peritoneum, invasion into the mesothelium, and survival and proliferation of the ectopic endometrial cells.

MicroRNAs (miRNAs), members of a class of small noncoding RNA molecules, have a critical role in posttranscriptional regulation of gene expression by repression of target mRNAs translation. Originally identified in *Caenorhabditis elegans* [25], miRNAs have been shown to operate in a wide

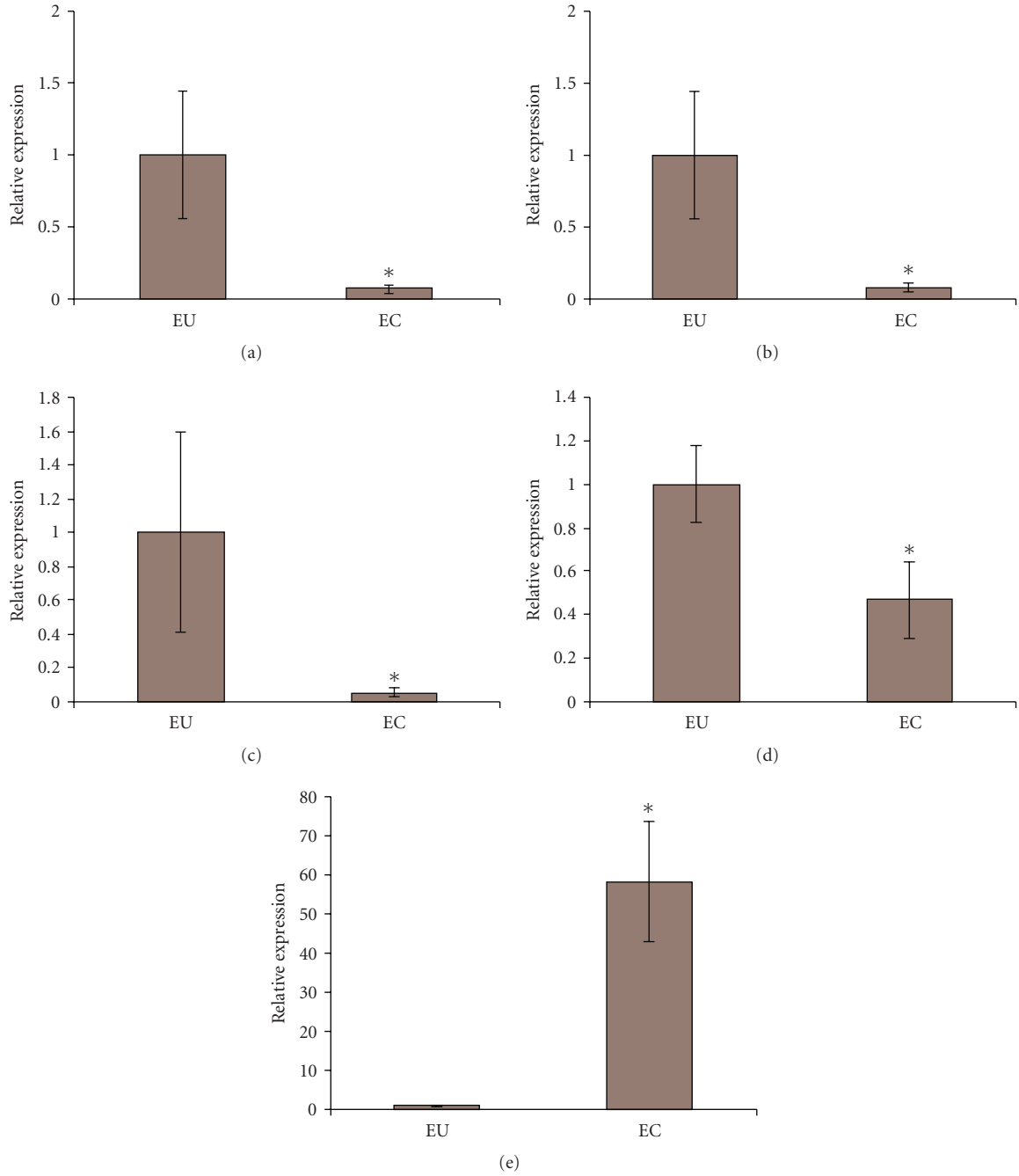

Figure 1: Differential expression of miRNAs in ectopic *versus* eutopic endometrium. The miRNAs selected for independent validation were among those with wider difference in expression between eutopic and ectopic endometrium. The differential expression of the selected miRNAs in the ectopic *versus* eutopic tissues was evaluated by real-time RT-PCR. The expression of each miRNA in eutopic tissue was set to 1. (a) hsa-miR-200a, (b) hsa-200b, (c) hsa-miR-200c, (d) hsa-miR-182, and (e) hsa-miR-202. $^*P < .05$.

range of species, including humans. Computational predictions indicate that up to 30% of human genes are potential targets of miRNAs and that miRNAs compose 1%–5% of animal genomes [26–29]. MiRNA expression is tissue- and cell-specific [30–33]. It has been demonstrated that miRNAs are important in developmental processes as well as for other cellular activities involving cell growth, differentiation, and apoptosis. Moreover, several genes encoding miRNAs have been located at chromosomal fragile sites or regions of cytogenetic abnormalities associated with cancer and other disorders. Interestingly, miRNAs altered expression has been associated with tumorigenesis, and several studies have described differential expression of miRNAs in neoplastic *versus* normal tissue [34–38].

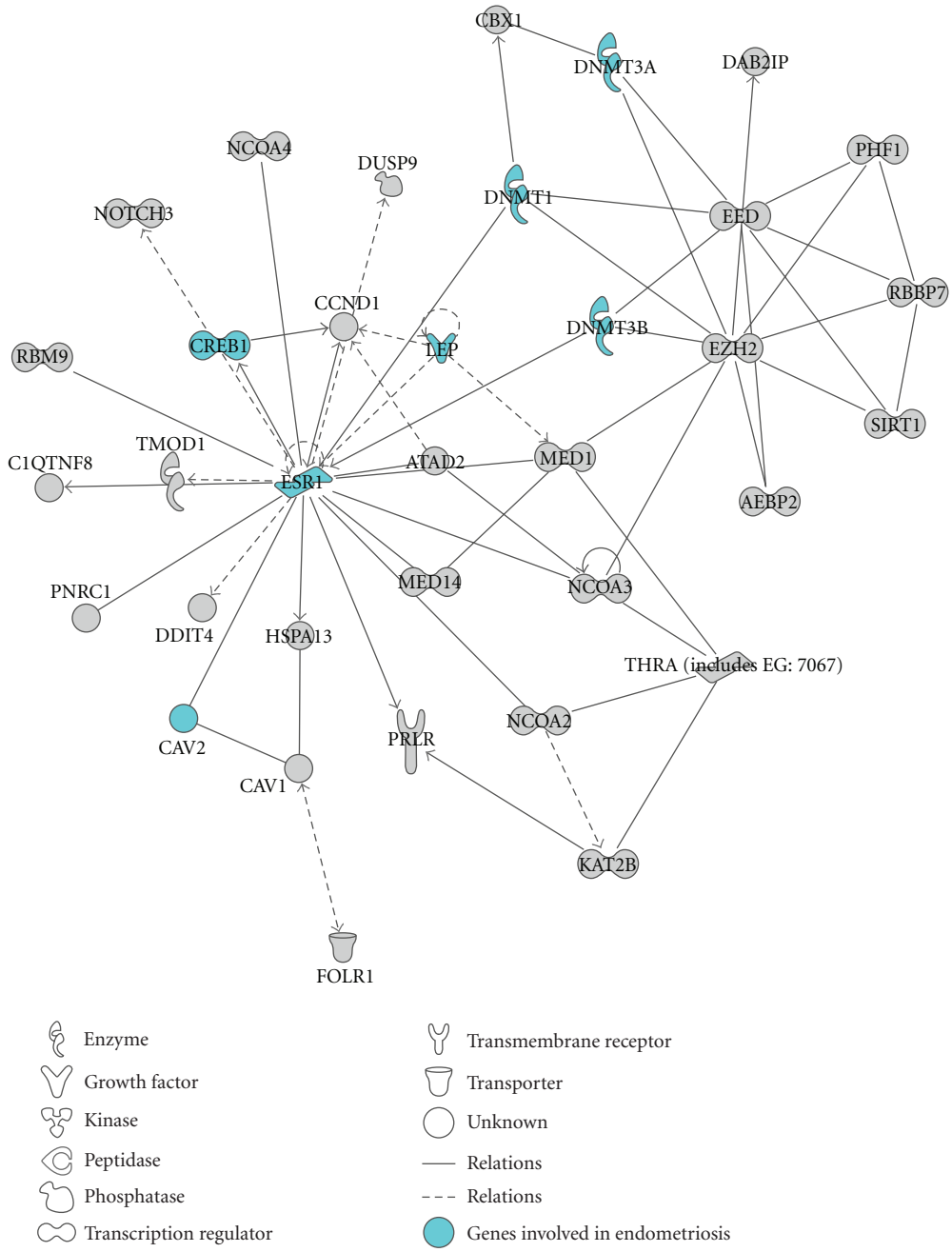

Figure 2: Functional analysis of all the predicted targets of the differentially expressed miRNAs. Graphical representation of network #2 obtained by IPA analysis. Genes are represented as nodes, and the biological relationship between two nodes is represented as a line. Every line is supported by at least one reference in literature. Highlighted, the genes involved in endometriosis according to IPA knowledge base.

Our study is aimed to investigate the differential expression of miRNAs in endometriosis by direct comparison between paired ectopic and eutopic endometrium samples. Once we identified the differentially expressed miRNAs, we validated 5 of them by an independent technique. Then, we identified *in silico* the predicted molecular targets of the differentially expressed miRNAs and we used a bioinformatics tool to investigate the molecular pathways in which these targets could be involved.

2. Materials and Methods

2.1. Tissue Collection. Subjects ($n = 16$) scheduled for surgery for chronic pelvic pain or infertility at the University of Piemonte Orientale-affiliated "Maggiore della Carità" Hospital were recruited to participate in this study. The study was approved by the "Maggiore della Carità" Hospital's Institutional Review Board and informed consent was obtained from all participants. None of the authors have any conflict

Table 1: Differentially expressed miRNAs in ectopic *versus* eutopic endometrium. List of differentially expressed miRNAs whose expression value in ectopic endometrium was at least twofold higher or lower than in eutopic endometrium $P < .01$.

Name	EU	EC	*P*-value
hsa-miR-1	36.29	2,090.27	.00E+00
hsa-miR-100	7,517.73	18,712.43	.00E+00
hsa-miR-101	341.51	2,348.69	.00E+00
hsa-miR-106a	3,264.74	1,510.10	1.11E−16
hsa-miR-106b	2,996.55	1,414.14	.00E+00
hsa-miR-126	10,373.88	22,435.79	.00E+00
hsa-miR-130a	1,634.84	5,145.94	.00E+00
hsa-miR-130b	673.04	249.86	.00E+00
hsa-miR-132	3,699.14	1,261.33	.00E+00
hsa-miR-143	8,104.26	21,764.97	.00E+00
hsa-miR-145	10,992.36	27,550.33	.00E+00
hsa-miR-148a	2,623.73	6,507.58	.00E+00
hsa-miR-150	1,621.96	4,503.15	.00E+00
hsa-miR-17-5p	4,517.66	2,059.32	.00E+00
hsa-miR-182	1,998.92	230.69	.00E+00
hsa-miR-183	410.83	41.02	.00E+00
hsa-miR-186	56.69	246.79	1.21E−14
hsa-miR-196b	380.45	14.13	.00E+00
hsa-miR-199a	4,481.27	12,618.11	.00E+00
hsa-miR-200a	582.95	33.22	.00E+00
hsa-miR-200b	17,643.11	675.98	.00E+00
hsa-miR-200c	25,249.55	1,391.63	.00E+00
hsa-miR-202	49.64	471.06	2.27E−13
hsa-miR-20a	5,278.72	2,534.21	9.05E−14
hsa-miR-221	5,368.05	10,915.55	.00E+00
hsa-miR-25	12,878.14	6,328.31	1.06E−14
hsa-miR-28	1,465.55	4,589.04	.00E+00
hsa-miR-299-5p	202.34	452.17	5.18E−13
hsa-miR-29b	248.51	4,963.66	.00E+00
hsa-miR-29c	295.40	10,562.63	.00E+00
hsa-miR-30e-3p	299.19	1,003.48	1.50E−14
hsa-miR-30e-5p	58.94	428.59	.00E+00
hsa-miR-34a	337.65	861.73	.00E+00
hsa-miR-365	264.57	2,071.70	.00E+00
hsa-miR-368	297.52	1,882.43	.00E+00
hsa-miR-375	1,329.85	13.62	.00E+00
hsa-miR-376a	64.20	522.49	.00E+00
hsa-miR-379	175.21	601.12	7.19E−13
hsa-miR-411	62.72	215.67	5.92E−16
hsa-miR-425-5p	961.30	329.99	.00E+00
hsa-miR-486	2,824.50	956.89	.00E+00
hsa-miR-493-5p	64.60	355.15	7.22E−12
hsa-miR-503	2,084.95	465.94	.00E+00
hsa-miR-638	29,531.60	11,202.65	.00E+00
hsa-miR-663	4,654.42	1,943.14	4.44E−15
hsa-miR-671	2,052.70	955.73	.00E+00
hsa-miR-768-3p	5,841.89	2,901.78	2.81E−06
hsa-miR-768-5p	5,321.54	2,456,43	.00E+00
hsa-miR-93	2,614.63	629.20	.00E+00
hsa-miR-99a	6,766.02	18,369.57	.00E+00

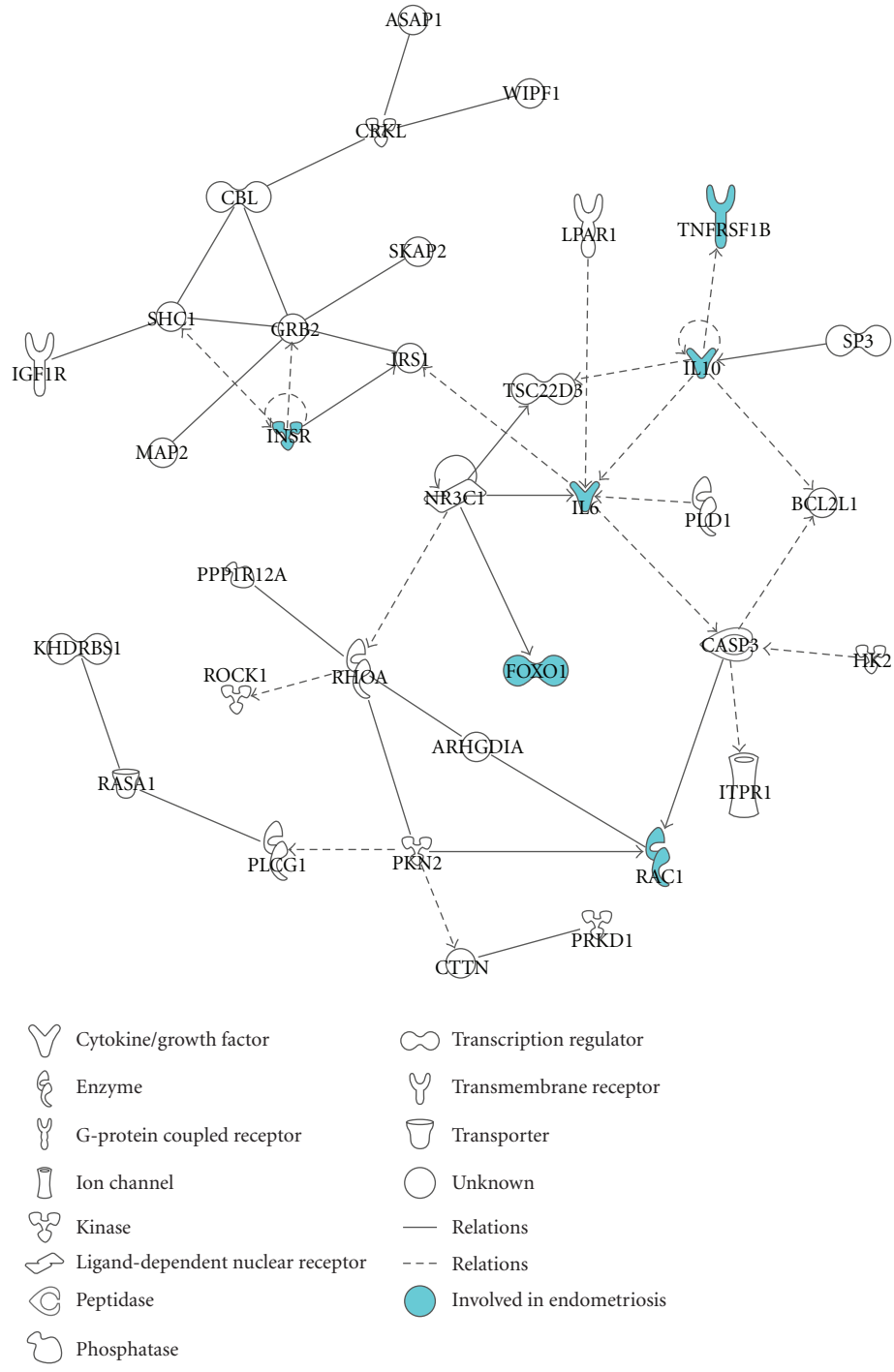

Figure 3: Functional analysis of the predicted targets of miR-182, miR-200a, miR-200b, miR-200c, and miR-202 identified by Pictar and Targetscan: graphical representation of one of the network (P-value = 10E−37, focus molecules = 35) identified by IPA analysis of the predicted targets of the miRNAs whose differential expression in eutopic and ectopic endometrium was validated by real-time RT-PCR. Highlighted are the genes involved in endometriosis according to IPA knowledge base.

of interest with the study. Surgery was scheduled 6 to 12 days after the onset of menses. No patients were receiving hormone therapy at the time of the study or in the previous three months. The patients ranged in age from 24 to 48 years, with an average of 36 years. Endometriomas were removed at laparoscopy by excision of the entire cyst wall by stripping technique, preserving normal ovarian tissue. Hysteroscopy with directed biopsies, performed to obtain a sample of eutopic endometrium from the same patient, were carried out using a 4 mm Bettocchi Hysteroscope System with a

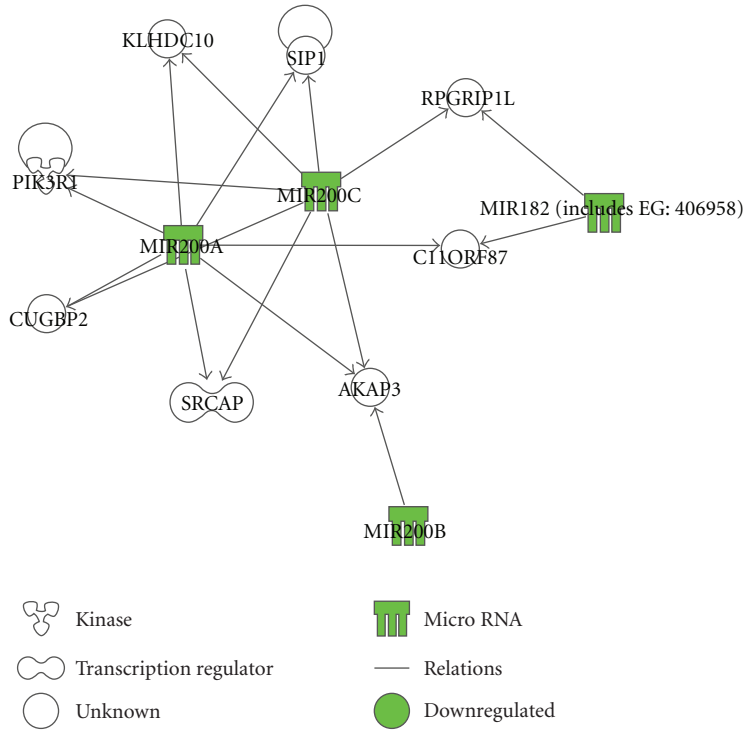

Figure 4: Functional analysis of the predicted targets of miR-182, miR-200a, miR-200b, miR-200c, and miR-202 identified by Argonaute2: graphical representation of the network identified by IPA analysis of the miRNAs and their predicted targets using the database generated by Argonaute2 algorithm (P-value = 10E−14, focus molecules = 4).

5 Fr operative channel (Karl Stortz GmbH & Co., Tuttlingen, Germany). Laparoscopy and hysteroscopy procedures were performed during the same surgical intervention. Freshly recovered tissues were rinsed in saline solution and divided in two parts. One half of the tissue was immediately snap-frozen and kept in liquid nitrogen for further processing, while the other was sent to the pathology laboratory. The endometriomas of 9 patients were classified as moderate, while 7 were classified as severe according to the ASRM guidelines [39].

2.2. RNA Isolation. Total RNA was extracted from tissues with the miRNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and quantified by Quant-iT RNA Assay Kit with Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA).

2.3. MicroRNA Microarray Assay and Analysis. Microarray assay was performed using a service provider (LC Sciences). Ten μg of total RNA from eutopic and ectopic endometrium obtained from three patients were size fractionated using a YM-100 Microcon centrifugal filter (Millipore) and the small RNAs (<300 nt) isolated were 3′-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a μParaflo microfluidic chip using a microcirculation pump (Atactic Technologies) [40, 41]. On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target 475 mature human miRNA probes (Sanger miRBase sequence database 9.1) or other RNAs for control and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by in situ synthesis using PGR (photogenerated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 μL 6xSSPE buffer (0.90 M NaCl, 60 mM Na_2HPO_4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C. After RNA hybridization, tag-conjugating Cy3 and Cy5 dyes were circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data from miRNA microarray were analyzed by the service provider first subtracting the background and then normalizing the signals using an LOWESS filter (Locally weighted Regression) [42]. The ratio of the two sets of detected signals (log2 transformed, balanced) and P-values of the t-test were calculated; differentially detected signals were those with less than .01 P-values. Multiple sample analysis involved normalization, data adjustment, t-test, and clustering. Normalization was carried out using a cyclic LOWESS. Data adjustment included data filtering, Log2 transformation, and normalization. The t-test was performed between "control" and "test" sample groups

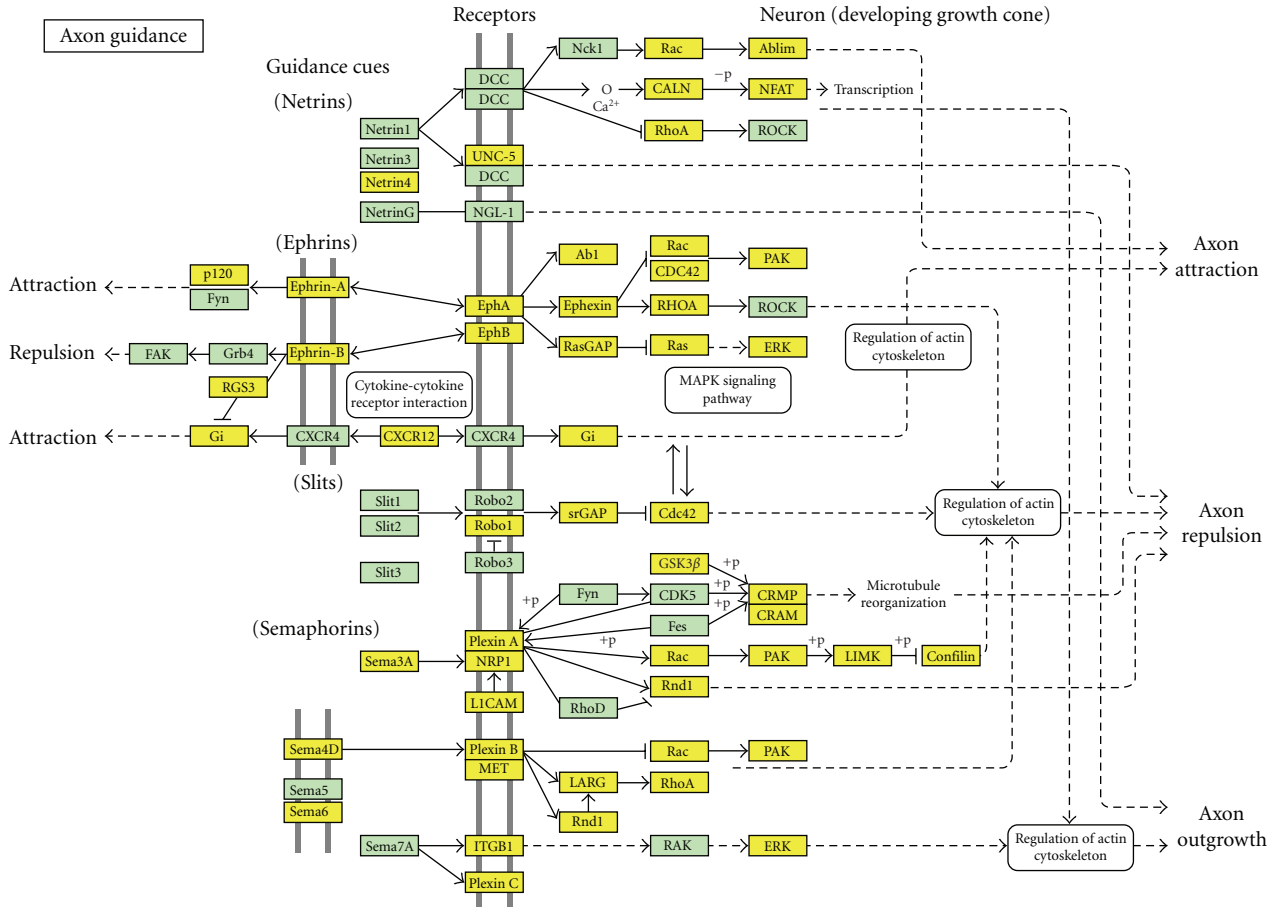

FIGURE 5: The axon guidance pathway identified by Pathway-Express analysis. Pathway-Express analysis performed on the predicted targets of the 50 differentially expressed miRNAs identified, among the most significant KEGG pathways predicted to be relevant for endometriosis, the *axon guidance* pathway. In yellow are the predicted targets of the differentially expressed miRNAs.

[43]. *T*-values were calculated for each miRNA, and *P*-values were computed from the theoretical *t*-distribution. miRNAs with *P*-values < .01 were selected for cluster analysis. The clustering was done using hierarchical method and was performed with average linkage and Euclidean distance metric [44] using TIGR MultiExperiment Viewer (http://www.tm4.org/mev.html).

2.4. Reverse Transcription and Real-Time PCR. Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) was performed to confirm the differential expression of selected miRNAs, identified as differentially expressed by miRNA microarray, in paired samples from other 13 patients. TaqMan MicroRNA RT Kit (Applied Biosystems, Foster City, CA) was used for reverse transcription. Real-time RT-PCR reactions were carried out with a 7300 Real-Time PCR System (Applied Biosystems) according to the protocol provided by the supplier, using the TaqMan Universal PCR Master Mix No AmpErase UNG and the following TaqMan MicroRNA Assays: hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, hsa-miR-182, hsa-miR-202, and U18 as endogenous control.

Data from real-time RT-PCR experiments are presented as the mean ± SEM. The variation among groups was compared by means of nonparametric Wilcoxon and Mann-Whitney *U* tests. Statistical significance was assumed for *P*-values < .05. Statistical analysis was performed with SPSS for Windows version 15.0 (SPSS; Chicago, IL).

3. Results and Discussion

3.1. MicroRNAs Differentially Expressed in Eutopic and Ectopic Endometrial Tissue. In the present study, we used miRNA microarray technology to identify the pattern of miRNAs in paired eutopic/ectopic endometrium from the same patients, thus avoiding the variables attributable to heterogeneous genetic background between individuals and the effects of estrogenic stimulation during different phases of the menstrual cycle. Moreover, we considered the whole endometrial and endometriotic tissues in order to preserve the contribution of all the components of the tissues, including vascular and immune system components and to avoid potential changes in gene and miRNA expression due to cell isolation and manipulation.

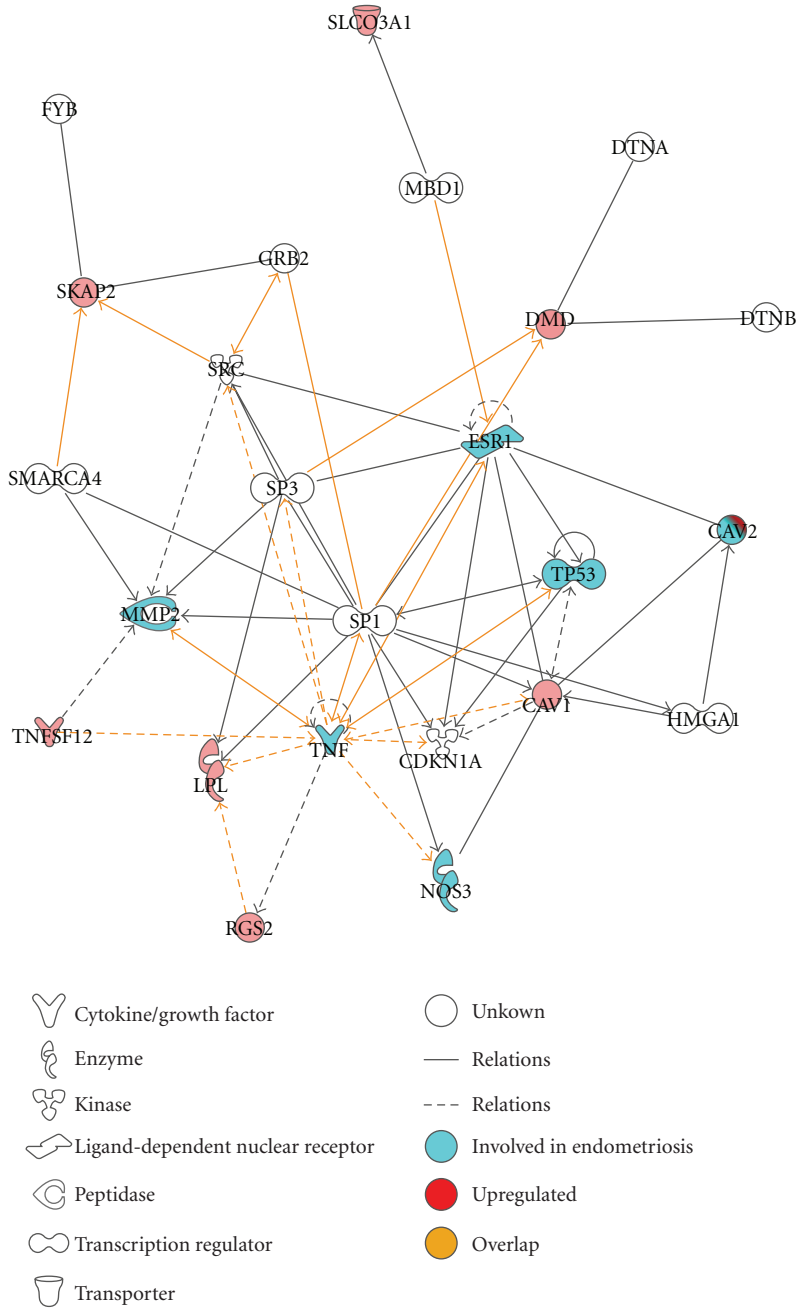

Figure 6: Functional analysis of the differentially regulated genes common in ovarian and peritoneal endometriosis that are predicted targets of the 50 miRNAs: Graphical representation of the overlap of the networks identified by IPA with, highlighted are the genes involved in endometriosis according to IPA knowledge base.

Microarray technology has allowed a global analysis of all miRNAs differentially expressed in ectopic *versus* eutopic endometrium. The initial analysis of miRNA expression in ectopic endometrium compared with eutopic endometrium of three patient samples generated a list of 84 miRNAs significantly differentially expressed (P-values $< .01$). The 50 miRNAs for which the expression value in ectopic endometrium was at least twofold higher or lower than in eutopic endometrium are listed in Table 1.

3.2. Real Time RT-PCR Analysis of miRNA Expression. In order to confirm the results obtained with miRNA microarray, the expression analyses of 5 selected miRNAs was carried out by real-time RT-PCR on specimens from other 13 patients. These 5 miRNAs, namely, hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, hsa-miR-182, and hsa-miR-202, were selected because their expression resulted to be highly altered in ectopic endometrium compared with the matched eutopic tissue. We verified the differential expression of the

Table 2: Molecular networks constituted by the predicted miRNA targets. IPA analysis was performed in order to identify the molecular pathways and functions to which the predicted targets of the differentially expressed miRNAs belong. The networks are generated on the basis of the published literature and ranked by the *P*-value calculated by Fisher's exact Test. The biological processes in which the targets are involved are determined by IPA using the GOstat application $P < .01$.

ID	Molecules in network	*P*-value	Focus molecules	Top functions
1	ACTR1A, ADM, APP, BICD2, CABP7, CELSR1, CPSF6, DAG1, ELAVL1, EPHA2, GCH1, GNA13, HIRA, HLX, HNRNPH1, HNRNPM, IFNG, IRF2, KHDRBS1, LARGE, MAPT, MTMR3, MTMR4, MYH9, PCSK2, PLCG1, PTGS2, RASA1, SBF1, SOCS1, SOCS2, STAT6, TNPO1, TNPO2, TRIB2	10E−21	35	Cellular Development, Skeletal and Muscular Disorders, Organismal Development
2	AEBP2, ATAD2, C1QTNF6, CAV1, CAV2, CBX1, CCND1, CREB1, DAB2IP, DDIT4, DNMT1, DNMT3A, DNMT3B, DUSP9, EED, ESR1, EZH2, FOLR1, HSPA13, KAT2B, LEP, MED1, MED14, NCOA2, NCOA3, NCOA4, NOTCH3, PHF1, PNRC1, PRLR, RBBP7, RBM9, SIRT1, THRA (includes EG:7067), TMOD1	10E−21	35	Gene Expression, Cellular Growth and Proliferation, Developmental Disorder
3	AKAP13, BCL2L11, CCNE2, CDK6, CDKN1A, CDKN1B, CTGF, CTSB, CUGBP1, DUSP1, E2F1, ESRRG, ETS1, FHL2, FLI1, FOXO1, FOXO3, FOXO4, IGFBP3, IP6K3, JAG1, KRAS, MCF2, NR3C1, PRKD3, RB1CC1, SGK1, SMAD3, SP1, SPHK2, TCF7L2 (includes EG:6934), TGFBR1, TIMP3, TOPBP1, TSPYL2	10E−21	35	Cellular Growth and Proliferation, Cellular Development, Cancer
4	ADAM12, BCL2, CITED2, EGLN1, FGF9, FRAP1, GATA3, GNAI2, HIF1A, HSPD1, IGF1R, IKBKB, ITGA9, JUNB, KPNA1, KPNB1, MAP2K3, MAP2K5, MAP3K7, MAP3K7IP2, PIAS3, PPM1B, PRKCE, PTEN, PTPN1, RPS6KB1, SKI, SMAD7, SNAI1, SOCS3, SP2, STAT3, UBR5, WT1, ZEB1	10E−21	35	Cellular Growth and Proliferation, Cellular Movement, Cell Cycle
5	ANP32A, ATP2A2, CD69, CDK5R1, COL1A1, COL1A2, CREM, DDR1, DLL4, E2F3, EGR1, FBXW7, FLT1, HDAC4, IL2, IL18BP, LPL, NDRG1, NOTCH1, PHC1, PHC2, POLA1, PPARA, RANBP2, RB1, RYBP, SHC1, SP3, SP4, TRAM2 (includes EG:9697), XPO1, YBX1, YY1, ZBTB10, ZBTB7B	10E−21	35	Organismal Injury and Abnormalities, Cardiovascular Disease, Cellular Development

Table 2: Continued.

ID	Molecules in network	*P*-value	Focus molecules	Top functions
6	ARNT, BACH1, BCL2L12, BRCA1, CLOCK, CREBBP, CYP1B1, DDX5, EP300, EPAS1, ERBB4, EREG, GABPA, GADD45A, HBEGF, HOXA13, HOXB6, LEF1, MAB21L1, MAX, NCAM1, NFYA, NPAS2, OXTR, PIN1, PPARG, PPP2CA, PTGER4, RBBP8 (includes EG:5932), RUNX1, SDC1, SLC1A2, TGFA, TRERF1, WNT5A	10E−21	35	Gene Expression, Cancer, Genetic Disorder
7	ACTB, ARID1A, ARID1B, ARID4A, ARID4B, BTG2, CLIP1, DR1, ETS2, EWSR1, GTF2B, HOXA9, PFN1, RARB, RBL1, RBL2, SAP30, SAP130, SFPQ, SIN3A, SMARCA2, SMARCA4, SMARCB1, SMARCC1, SMARCC2 (includes EG:6601), SNIP1, SUMO1, TACC2, TAF4, TAF5, TAF12, TBP, TDG, TOP1, XPO6	10E−21	35	Gene Expression, Cellular Assembly and Organization, Cellular Compromise
8	ARHGDIA, BTRC, CASP3, CD4, CDC42, CLTC, CTTN, CXCL12, DIABLO, ELK1, ELK3, EZR, F3, FOS, FOSB, GLI3, GSK3B, HNRNPA1, HNRNPC, IL1A, ITPR1, JUND, MAP1B, MCL1, OCRL, PAK1, PGM1, PRKCI, PRKD1, PTX3, RABEP1, RAC1, SEMA3A, SRF, STK4	10E−21	35	Cell Death, Cancer, Cellular Assembly and Organization
9	CD47, CSF1, CSF1R, CSK, EPHA4, FASLG, FGF1, FN1, FOXP1, GRB2, IRS2, ITGA5, ITGA6, ITGA10, ITGA11, ITGAV, ITGB1, ITGB3, JAK2, KCNA3, MAP2K1, MAP2K4, MAPK1, MET, MITF, NFAT5, PDGFB, PDGFRB, PLXNB1, RAB21, SERPINE1, TNFRSF1A, TNFSF11, TRIB1, YES1	10E−21	35	Cellular Growth and Proliferation, Cell-mediated Immune Response, Cellular Movement
10	ACTR3, AR, ARHGEF7, ASAP1, CRKL, DYRK1A, ESR1, GDI1, KLF2, LMOD1, LRRK1, MRAS, NCK1, PFTK1, PLS3, POMT2, TEAD3, TRIP10, WAS, WEE1, WIPF1, ZMIZ1	10E−9	19	Cellular Assembly and Organization, Skeletal and Muscular System Development and Function, Cancer
11	AKAP12, AMOTL2, ARL6IP1, ATM, BRCA1, CDC6, CHEK2, E2F1, FKBP3, HS3ST1, LATS2, LBR, MBNL2, MTDH, PPM1D, PPP1R13B, SCN3B, SH3BP4, TP53, TRIO, VCAN	10E−7	17	Cancer, Genetic Disorder, Reproductive System Disease
12	ANK3, CREB5, DEDD, FRK, GPRC5A, KCNK2, KRT18, MPZL2, MYCBP2, MYO1B, NRK, RAB22A, SPAG9, ZNF217	10E−7	13	Cardiovascular Disease, Cellular Development, Cell Morphology

Table 2: Continued.

ID	Molecules in network	*P*-value	Focus molecules	Top functions
13	ADAM19, CADM1, CBFA2T3, CDC42SE1, COL6A3, COL7A1, DAAM1, ERBB2, FN1, HAS3, MFAP2, MPHOSPH9, NET1, PMEPA1, RAP1B, TGFB1, THBS1, THPO, XYLT1, ZFP36	10E−7	16	Cancer, Cellular Growth and Proliferation, Dermatological Diseases and Conditions
14	ATP1B3, CCND1, COL3A1, COL4A1, COL5A2, CTNNB1, HOXA1, IGF2R, KLF9, LGALS3, M6PR, MAP3K10, NANOG, NPTX1, NRF1, NRIP1, PTPRC, PTTG1, RB1, SPTBN2, TCF7L2 (includes EG:6934), THRB (includes EG:7068), TP53	10E−6	17	Organismal Development, Cancer, Cell Cycle
15	ALDH1A3, COLQ, DUSP10, EIF4B, GPD2, HSPE1, IL6, IL13, IL1B, MMD, NR4A3, NUAK1, PTPN12, RND3, ROBO1, SEMA3C, SLC7A1, STAC, TNF, TNFSF10, TUB	10E−6	16	Small Molecule Biochemistry, Skeletal and Muscular System Development and Function, Cell-To-Cell Signaling and Interaction
16	ACSL3, ASXL1, EGR3, JMJD1C, PLK2, PTP4A1, RRM2, RRM1 (includes EG:6240), RRM2B, SEL1L, SFRS3, SLC2A1, SMURF2, SON, STRN3, TNF, TP53, UBE2B, ZFP36L1	10E−6	15	Nucleic Acid Metabolism, Small Molecule Biochemistry, Genetic Disorder
17	CCND1, CCNT1, CCNT2, CDK9, CDKN1A, DNAJB9, FBXW11, GLI1, GLI2, GNAO1, GTF2F2, HSPA5, HTATSF1, ID2, JAG2, MDFIC, MXI1, MYCN, NPM1 (includes EG:4869), POLR2A, POLR2C, RB1, RPS6KA1, RXRA, SFRS1, SUPT5H, SUPT6H, TCERG1, TGFB1, TP53, ULK1	10E−6	20	Gene Expression, Cellular Development, Cell Cycle
18	APBB2, BECN1, CAD, CDKN1A, CFL1, E2F5, ESR1, FANCA, FANCC, FGF7, GFI1, GJA1, GORASP2, HSP90AA1, LIMK1, MAX, MYC, PCBP2, PERP, PTBP1, SPTAN1, TERT, TMSB4X, TP53, XBP1	10E−5	17	Cell Cycle, Connective Tissue Development and Function, Cellular Compromise
19	AP3M1, BCL6, CCND1, CREBL2, ENC1, FOXA1, FTH1, HNF1A, HNMT, MTA3, MUC4, NCOR1, NCOR2, NFE2L2, NFYC, NR5A2, SNX17, SSTR1, TFR2, TFRC, TMOD2	10E−5	15	Cancer, Gene Expression, Drug Metabolism
20	ACTB, ACTL6A, CCNT1, CD9, CTCF, DMAP1, EMD, EPC1, ESR1, HABP2, HNRNPA1, HNRNPF, HNRNPK, HSP90AA1, LEMD3, MKNK2, MORF4L1, MYC, PCBP1 (includes EG:5093), SYNE2, TBP, THOC4, TNPO1, TRRAP, U2AF1, WNT1, WNT2B, YY1, ZBTB33	10E−5	18	Gene Expression, Cancer, Reproductive System Disease

Table 2: Continued.

ID	Molecules in network	*P*-value	Focus molecules	Top functions
21	BEX2, CDH1, CDH2, CDH11, CTNNA2, CTNNB1, CTNND2, DIO2, ELAVL1, EPHB3, ERBB2, ESR1, F13A1, HNRNPD, ILF3, IRS1, JUP, KHSRP, LDB1, LMO2, NHLH2, PIK3R1, PKD1, PPP3CA, PTCH1, PTPRF, TCF7L2 (includes EG:6934), TIAL1, TP53, TSC22D1, ZNF346	10E−4	18	Cell-To-Cell Signaling and Interaction, Cancer, Cellular Growth and Proliferation
22	ACIN1, AP2A1, BRD2, COIL, EIF4A1, EIF4G3, ICMT, LMO7, MAP7, NME1, PA2G4, PABPC1, PABPN1, PAIP1, PAIP2, PAPOLG, PNN, RNGTT, RNPS1, SAP18, SFRS11, TALDO1, TRA2B, ZNF143	10E−4	15	RNA Post-Transcriptional Modification, Protein Synthesis, Gene Expression
23	ADAM9, ADAM10, BMP7, CCL2, CCL5, CDH1, COL18A1, CTNNB1, DICER1, EGF, EGFR, EPS15, ERBB2, ETV1, GRB2, HGS, IL8, L1CAM, LPAR1, LPP (includes EG:4026), MAP3K14, NKRF, RALA, RELA (includes EG:5970), SHC1, SMAD5, SPG20, SRC, TBK1, TERT, TJP1, TMEM55A, TMEM55B, TNF	10E−4	19	Cell-To-Cell Signaling and Interaction, Tissue Development, Cancer
24	ALOX15, BZW2, CCL3, CHST2, DHCR24, FCER2, GAS7, GATA6, IGHE, IL4, IL8, IL13, MTSS1, NHLH1, NOS2, NOTCH2, PDGFC, PHLDA1, PLXNC1, RIN2, SORT1, ST8SIA4	10E−4	14	Genetic Disorder, Inflammatory Disease, Respiratory Disease
25	CAND1, CCND2, CDC5L, CDKN1B, CUL1, CUL2, CUL3, DNTT, FBXL3, FBXW2, GPR37, PARK2, PITX2, PLRG1, PMS1, PRCC, PRPF19, PSMA2, PSMC1, PSMC5, RAD23B, RBX1 (includes EG:9978), SFRS2, SKP1, SKP2, TCEB1, VHL	10E−4	16	Post-Translational Modification, Cancer, Immunological Disease
26	AMOT, B4GALT5, BTG3, CCL2, CD40, CHMP2B, CLASP1, ETS1, F3, FOS, HIVEP1, IKBKB, IL2, IL6, IL15, JAK1, JUN, MAPK1, MAPK14, MVP, NEFM, NFKB1, NFKBIA, PLG, PPP2R1B, RAB32, RELA (includes EG:5970), RFWD2 (includes EG:64326), RGS2, SQSTM1, STAT1, STAT3, TNF, TYK2, ZBTB11	10E−4	19	Hematological System Development and Function, Cell Death, Cell Cycle
27	CCNA2, CCNB1, CCNE1, CCNE2, CD46, CD59, CDK2, CDKN1C, E2F4, EPHB2, FBXO32, HDAC9, HIVEP2, IGFBP3, KLF4, LATS1, LTC4S, MYB (includes EG:4602), MYBL2, NDC80, NUMB, PCNA, PLAU, POLD1, RALBP1, RBL1, RBL2, RFC4, RFX1, SCD, SPARC, SUZ12, TGFB1, TGFB3, TNS3	10E−4	19	Cell Cycle, Cancer, Cellular Growth and Proliferation

Table 2: Continued.

ID	Molecules in network	*P*-value	Focus molecules	Top functions
28	ABL1, ADRB2, ATP1A1, ATP1A2, ATP1B1, BCAR1, BCAR3, BCR, CBL, CRK, DOCK1, FRAP1, FYN, GATA2, GRK4, ITGA2B (includes EG:3674), ITGB3, MAPK9, MGRN1, NEU2, PIAS1, PIK3R1, PLSCR1, PRKCD, PTK2, RAPGEF1, RECK, SP3, SRC, STAT1, TIMP2, TP73, TP53INP1, TSG101, VPS28	10E−4	19	Cell Death, Cellular Movement, Cellular Growth and Proliferation
29	AKAP11, B2M, BHLHE40, CALD1, CEBPA, CHI3L1, COL16A1, EDN1, EDNRB, EIF1AX, EMP1, HMGA1, HMGCR, HMGCS1, IDI1, INSIG1, IPO13, KIT, KITLG, LSS, MMP2, MMP3, NAMPT, NPPB, PRKCA, PTPN6, RETN, SCARB1, SERPINB1, SERPINE1, TGFBR2, TGIF2, TNC, TNF, UBE2I	10E−4	19	Cancer, Hematological Disease, Lipid Metabolism
30	ALOX12B, APOE, ATF7IP, BCAT2, CAMK2A, CAMK2N1, CCND3, CDKN1B, CHAF1A, CRYBB2, DPP4, EFNA5, ESR1, GSTP1, HBE1, IL4, IL13RA1, IRF4, KCNK10, MBD1, MBD2, MBD3 (includes EG:53615), MECP2, MGMT, NR1H3, NR2F2, PIP5K3, PRLR, PTPN4, PTPRM, SETDB1, SLCO3A1, TFF2, TP53BP2, TSC1	10E−4	19	Behavior, Reproductive System Development and Function, Neurological Disease
31	ARHGEF6, BNIP2, CASP8, CHFR, CPD, CS, ELF1, IFNB1, IL8, IL16, INS, IRF1, JAK2, LMTK2, NCF2, NFKBIA, NGFR, PGAM1, PGK1, PLAGL2, PPP1CC, PPP1R12B, PRL, STAT1, TNF, TRADD	10E−3	15	Immunological Disease, Cell Death, Hematological Disease
32	ACHE, AGT, APP, ATP2B1, BACE1, BIK, BMP2, BTG2, CCL20, CD40LG, CDH1, CXCL2, CYCS (includes EG:54205), EFNA1, EIF4E, EIF4EBP1, GCLC, ITM2B, JUN, LAMP3, LYN, MYO6, PDK4, PPARD, PSEN1, PTGS2, PXN, SMAD1, SMPD2, SOX9, TNF, TNFAIP2, TNFSF10, TRPV6, VCL	10E−3	18	Cell Death, Cancer, Cell-To-Cell Signaling and Interaction
33	AHR, ANP32B, ATM, BIRC3, BTG2, CAMK2G, CEBPE, CLU, ELAVL1, ERCC1, GDF11, H2AFX, HDAC3, HNRNPD, HNRNPU, HOXA5, ILF3, NEDD8, NUP153, RAD50, RARA, RARB, RARG, TBX3, TERF2, TERF2IP, TIA1, TIAL1, TINF2, TP53, TPR, XPO1, XRCC5, XRCC6, YAP1	10E−3	18	Cell Cycle, DNA Replication, Recombination, and Repair, Cell Death

Table 2: Continued.

ID	Molecules in network	*P*-value	Focus molecules	Top functions
34	ADH5 (includes EG:128), ASH2L, ATP6V0C, C16ORF53, CHRNA5, CSNK2A1, CSNK2B, DPY30, EDA, ETV4, HCFC1, HDAC1, HIST2H4A, MIER1, MLL3, MLL4, MRC2, NCOA6, OGT (includes EG:8473), PAXIP1, PKNOX1, PLAU, PLAUR, POU2F1, RBBP5, SIN3A, SP1, SP3, SSRP1, SUB1, SUPT16H, TEAD1, TRIM63, WDR5, ZBTB7A	10E−3	18	Gene Expression, Cell Morphology, Reproductive System Development and Function
35	APLN, BID, CASP2, CFLAR, CXCL13, CYCS (includes EG:54205), DIABLO, EIF2S1, EIF4B, EIF4E, EIF4EBP1, EIF4G1, IL21, IL1RN, INHA, INHBA, INHBB, JAK1, LEFTY1, MCL1, NFKB2, P4HA1, PPP1R15A (includes EG:23645), PRDM1, SATB1, SERPINB2, SOCS1, SOCS3, SUV39H1, TAL1, TLR4, TNF, TNFSF10, USF1, USF2	10E−3	18	Protein Synthesis, Cancer, Cell Death
36	CEBPB, CSF1, CSF3, EGFR, FGA, GAB1, GRB2, IL6, IL1A, IL6ST, IRS1, JAK1, KIF5B, LIFR, LMO4, LPAR2, MAP2, MED28, NF2, NFKB1, OSM, OSMR, PIK3C2B, PLG, POU2F1, POU2F2, PRL, PTGS2, PTPN11, RNASE1, RNASE2, SKAP2, STAT3, TLR9, VIP	10E−3	18	Cellular Development, Cellular Growth and Proliferation, Cancer
37	AOF2, BAZ1A, BAZ1B, CACNA1C, CDYL, CHRAC1, CTBP1, CTBP2, EHMT1, EHMT2, GATA4, HAND1, HAND2, HDAC2, HMG20B, KCNJ3, MEF2C, MYOCD, PDS5A, PHF21A, POLE3, RAD21, RBBP4, RCOR1, RREB1, SCN5A, SFRP1, SMARCA1, SMARCA5, SMC3, SMC1A, STAG1, STAG2, WIZ, ZEB2	10E−3	18	Cell Cycle, DNA Replication, Recombination, and Repair, Gene Expression
38	AKAP1, API5, ARHGEF12, CFTR, COL18A1, F2, F2R, FGF2, FGFR1, IL1B, IQGAP2, MPRIP, PPP1R12A, PRKAR2B, PRKG1, PTGER3, RHOA, SH3GLB1, SH3GLB2 (includes EG:56904), SLC9A3R1, SRC, STX1A, VCP	10E−3	13	Cellular Assembly and Organization, Cell Morphology, Cancer
39	ACVR1, ACVR1B, ACVR2A, ANTXR1, APC, ASAP2, BCAP31, BIN1, BMP2, BMP6, BMP7, BMPR2, BMPR1A, CANX, COL18A1, CTNNB1, DCTN1, EFNB2, ERBB2, F10, ICAM1, ID1, ITGB2, MAPRE1, NOG, NRP1, PLP2, SEC23A, TGFB1, TLN1, TNFRSF21	10E−3	16	Cell Signaling, Cellular Development, Connective Tissue Development and Function

Table 2: Continued.

ID	Molecules in network	*P*-value	Focus molecules	Top functions
40	ARCN1, BRCA2, BRIP1, COPB1, COPG, CYLD, EXO1, HERC2, KPNA2, KPNB1, MAD2L2, MLH1, MMS19, MSH6, PIK3C2A, PMS1, PMS2, PSD2, PSMC1, RANBP9, REV1, REV3L, RFC2, RUFY1, SACM1L, SBF2, SSB (includes EG:6741), TMED9, UBA52, UBR5, USP5	10E−2	15	DNA Replication, Recombination, and Repair, Cancer, Gastrointestinal Disease
41	CCNB1, CD44, EGFR, EIF3A, ERBB2, ERRFI1, GAB1, IL6ST, JARID1B, KRT7, MYBL2, MYO10, NEDD9, PARP1, PIK3CA, PIK3CD, PIK3R1, PIK3R2, RAB31, SMAD2, SOLH, SOX4, TGFB1, TGIF1, TGOLN2 (includes EG:10618), TNF	10E−2	13	Cell Cycle, Cellular Growth and Proliferation, Carbohydrate Metabolism
42	ADCYAP1, AMPD3 (includes EG:272), CCL3, CCL4, CCL5, CD40, CD40LG, CSF3, CXCL10, DUSP1, DUSP6, FURIN, IER2, IL3, IL17A (includes EG:3605), IL1B, ITGAM, MAP2K6, MAPK3, MAPK14, MMP9, NAMPT, NFKB2, NGF, NR4A2, NSMAF, P2RX7, PLD1, PLG, PTGFR, SERPINB2, TOB1, TRAF3, TSC22D3, VEGFA	10E−2	16	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking
43	ATM, ATR (includes EG:545), C10ORF119, CDC6, CDC37, CDC25A, CDC25B, CHEK1, CHEK2, CSNK1A1, E2F1, FAS, GRB10, MAP3K11, MAP3K5 (includes EG:4217), MCM2, MCM3, MCM4, MCM7, MDM4, PLK1, PPP2R3A (includes EG:5523), PPP5C, RAD17, RAF1, SNAP23, SSH2, STX4, STX6, STX16, TP53, VAMP2, VAMP3, VIM, YWHAB	10E−2	16	DNA Replication, Recombination, and Repair, Cancer, Cell Cycle
44	BAK1, BAX, BCL2, BCL2L1, BID, BMF, BSG, CAV1, CAV3, CDC2, CDK2, CIT, CYCS (includes EG:54205), DLG4, ECT2, GIT1, GRIN2A, HINT1, HTT, IGFBP5, KIF14, KIF23, KRAS, LRP1, MEOX2, NCL, NCSTN, NT5C3, PLK1, PRC1, PSEN1, PSEN2, RACGAP1, TP53, VDAC2	10E−2	15	Cell Death, Cell Cycle, Cancer
45	ASCL2, ASF1A, ATXN7, CCNH, CDK7, CRIP2, CSPG4, DKK1, ENO3, ERCC2, ERCC3, ESRRA, GK, GPR64, GTF2H1, GTF2H2, HMGN1, MLL2, MNAT1, NR2C2, NT5E, PPARGC1A, RBBP5, SAFB, SMAD6, TAF1, TAF2, TAF4, TAF8, TAF9, TAF11, TAF15, TFF1, TUBB, UTX	10E−2	15	Gene Expression, DNA Replication, Recombination, and Repair, Dermatological Diseases and Conditions

Table 2: Continued.

ID	Molecules in network	*P*-value	Focus molecules	Top functions
46	ADAMTS5, BAX, BCL2, BCL2L1, BRCA1, CASP3, CCL3, CCL4, CD226, CD244, CSF2, FLNB, GP9, GP1BA, IL8, IL15, IL18, IL18R1, KLRK1, LCP2, MMP1 (includes EG:4312), MNT, MOAP1, NCR1, PDIA3, RAB9A, SELL, SOD2, TERT, TP63, VDAC1, XRCC6, YWHAE, YWHAQ (includes EG:10971), YWHAZ	10E−2	15	Cell-to-Cell Signaling and Interaction, Hematological System Development and Function, Cell Death
47	ABCA1, AKT1, APOA1, CCDC88A, CCL2, CCL5, COL2A1, CSH1, CUL5, FKBP1A, FLOT1, IGF1, IL8, IL13, IL1B, IL1RN, ILK, INS, LOX, MMP7, PDE4D, PDPK1, PGF, RNF4, RYR1 (includes EG:6261), SLC2A4, STK38L (includes EG:23012), TNF, TRPS1	10E−2	13	Cell-mediated Immune Response, Cellular Movement, Lipid Metabolism
48	EIF2C1, EIF2C2, TNRC6A	10E−2	3	Infection Mechanism, Cancer, Respiratory Disease
49	DMD, DTNA, DTNB	10E−2	3	Cellular Assembly and Organization, Nervous System Development and Function, Skeletal and Muscular System Development and Function

selected miRNAs in the ectopic tissue by setting as 1 the expression of eutopic miRNAs. The results obtained by real-time RT-PCR are in accordance with those obtained from the microarray. Indeed, these miRNAs showed significant differential expression (*P*-values < .05) in eutopic *versus* ectopic tissue: hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, and hsa-miR-182 levels in ectopic endometrium were reduced up to 95% (Figures 1(a)–1(d)), while hsa-miR-202 expression in ectopic endometrium was increased up to 60 folds compared to eutopic endometrium (Figure 1(e)). The analysis of data according to the severity of the endometrioma, by means of nonparametric Wilcoxon and Mann-Whitney *U* tests, failed to reveal any significant differences in miRNA expression levels, although this may be ascribable to the group size. Further studies increasing the cohort will be necessary to completely address this issue.

3.3. Identification of Predicted miRNA Targets and In Silico Functional Analysis. The predicted target mRNAs of the differentially expressed miRNAs common to two different search algorithms, TARGETSCAN (http://www.targetscan.org/) and PICTAR-VERT (http://pictar.mdc-berlin.de), were 3093. The functions of these predicted targets and the molecular pathways in which they could be involved were assessed using Ingenuity Pathways Analysis software (Ingenuity IPA 7.5). The predicted targets were uploaded in IPA, and the software identified 49 significant molecular networks to which the predicted targets of the differentially expressed miRNAs belong (Table 2). Among the biological functions reported to be statistically significant by IPA there were functions known to be involved in endometriosis such as gene expression, cellular growth and proliferation, cellular development, cellular movement, cell death, cell cycle, cancer, and reproductive system disorders. One of the subcategories of reproductive system disorders to be more represented, with *P*-value (calculated by Fisher's Exact test) of $6.1 \cdot 10^{-18}$, was endometriosis with 119 molecules directly involved in this pathology (Table 3).

An exemplificative network identified by IPA enriched for miRNA targets involved in endometriosis is shown in Figure 2. This network, converging on estrogen receptor 1 (ESR1), includes the DNA methyltransferases DNMT3A and DNMT3B that are validated targets of hsa-miR-29b and hsa-miR-29c, and of hsa-miR-29b, hsa-miR-29c, and hsa-miR-148a, respectively [45, 46]. DNA methylation is an epigenetic modification that is involved in gene silencing, chromatin remodeling, and genome stability [47]. It has been demonstrated that DNMT1, DNMT3A, and DNMT3B are disregulated in endometriosis [48], and it has been suggested that aberrant methylation of HOXA10 and of the progesterone receptor PR-B may be responsible of the disregulation of their expression in endometriosis. Thus, this network strongly suggests a possible involvement of miRNAs in these mechanisms.

Table 3: Molecules directly involved in endometriosis and networks in which they appear. IPA analysis indicated that several networks constituted by the predicted targets of the differentially expressed miRNAs include molecules known to be involved in endometriosis.

	Symbol	Entrez Gene Name	Networks
Cytokines	CD40LG	CD40 ligand	38
	CX3CL1	chemokine (C-X3-C motif) ligand 1	12
	CXCL13	chemokine (C-X-C motif) ligand 13	36
	IL2	interleukin 2	2, 29, 37
	IL4	interleukin 4	29, 32, 37
	IL6	interleukin 6 (interferon, beta 2)	29
	IL8	interleukin 8	28, 31
	IL18	interleukin 18 (interferon-gamma-inducing factor)	29
	SPP1	secreted phosphoprotein 1	23, 46
	TNF	tumor necrosis factor (TNF superfamily, member 2)	26, 29, 30, 33, 37, 38, 40, 41, 45
Enzymes	CNTN1	contactin 1	23
	DNMT1	DNA (cytosine-5-)-methyltransferase 1	5, 36
	DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha	5
	DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta	5
	FN1	fibronectin 1	7, 28
	GNAS	GNAS complex locus	41
	GSTP1	glutathione S-transferase pi 1	49
	HINT1	histidine triad nucleotide binding protein 1	48
	KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	3
	PDE4A	phosphodiesterase 4A, cAMP-specific (phosphodiesterase E2 dunce homolog, Drosophila)	44
	PDE4D	phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	41
	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	7, 28
	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	5
	RAP1B	RAP1B, member of RAS oncogene family	23
	REV3L	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	39
	RRM1 (includes EG:6240)	ribonucleotide reductase M1	26
	SAT1	spermidine/spermine N1-acetyltransferase 1	26
	XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6	42, 48

Table 3: Continued.

	Symbol	Entrez Gene Name	Networks
Growth Factors	ANGPT2	angiopoietin 2	7
	CTGF	connective tissue growth factor	2, 36, 40
	FGF2	fibroblast growth factor 2 (basic)	23, 31
	INHBA	inhibin, beta A	45
	LEP	leptin	6
	TGFB1	transforming growth factor, beta 1	20, 26, 33, 35, 40, 45
	VEGFA	vascular endothelial growth factor A	29, 30
Ion Channels	PKD1	polycystic kidney disease 1 (autosomal dominant)	45
	PKD2 (includes EG:5311)	polycystic kidney disease 2 (autosomal dominant)	45
Kinases	CDC2	cell division cycle 2, G1 to S and G2 to M	32, 36
	CSF1R	colony stimulating factor 1 receptor	3, 34
	EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	28
	ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	27, 30, 33, 35, 38, 40, 45, 47
	FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	2
	INSR	insulin receptor	26
	JAK1	Janus kinase 1 (a protein tyrosine kinase)	37
	KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	9
	MAPK4	mitogen-activated protein kinase 4	17
	NTRK2	neurotrophic tyrosine kinase, receptor, type 2	45
	PCK1	phosphoenolpyruvate carboxykinase 1 (soluble)	42
	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	11
	PDGFRB	platelet-derived growth factor receptor, beta polypeptide	11
	PIK3R2	phosphoinositide-3-kinase, regulatory subunit 2 (beta)	17
	SGK1	serum/glucocorticoid regulated kinase 1	16
	STC1	stanniocalcin 1	49
	WEE1	WEE1 homolog (S. pombe)	18
Ligand-Dependent Nuclear Receptors	AHR	aryl hydrocarbon receptor	44
	AR	androgen receptor	30
	ESR1	estrogen receptor 1	5, 30, 44
	ESR2	estrogen receptor 2 (ER beta)	44

Table 3: Continued.

	Symbol	Entrez Gene Name	Networks
	PPARG	peroxisome proliferator-activated receptor gamma	12, 29
Peptidases	HPR (includes EG:3250)	haptoglobin-related protein	24
	MEST	mesoderm specific transcript homolog (mouse)	18
	MMP2	matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	11
Phosphatases	DUSP1	dual specificity phosphatase 1	3
	PPP3R1	protein phosphatase 3 (formerly 2B), regulatory subunit B, alpha isoform	24
	PTEN	phosphatase and tensin homolog	19
	PTP4A1	protein tyrosine phosphatase type IVA, member 1	22
Transcription Regulators	BCL6	B-cell CLL/lymphoma 6	16
	BRCA1	breast cancer 1, early onset	5, 30, 42
	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	11
	CREB1	cAMP responsive element binding protein 1	6
	EGR1	early growth response 1	6, 26, 35
	EMX2	empty spiracles homeobox 2	15
	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	6, 35, 49
	FOXO1	forkhead box O1	3
	GATA3	GATA binding protein 3	2
	HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	10, 31, 44, 47
	ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	47, 50
	JUN	jun oncogene	49
	JUNB	jun B proto-oncogene	11, 47
	NRIP1	nuclear receptor interacting protein 1	36
	REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	44
	SMAD6	SMAD family member 6	14
	SMAD7	SMAD family member 7	11
	SP2	Sp2 transcription factor	31
	TP53	tumor protein p53	22, 27, 32, 34, 36, 37, 40, 41, 45
	WT1	Wilms tumor 1	7
	ZFP36	zinc finger protein 36, C3H type, homolog (mouse)	20
Transmembrane Receptors	IL2RG	interleukin 2 receptor, gamma (severe combined immunodeficiency)	37
	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	11, 28, 30
	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	7

Table 3: Continued.

	Symbol	Entrez Gene Name	Networks
	ITGB4	integrin, beta 4	30
Transporters	APOE	apolipoprotein E	36
	ATP1B1	ATPase, Na^+/K^+ transporting, beta 1 polypeptide	41
	ATP2B2	ATPase, Ca^{++} transporting, plasma membrane 2	21
	SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	1
Others	ACTB	actin, beta	9, 49
	ANK3	ankyrin 3, node of Ranvier (ankyrin G)	24
	BCL2	B-cell CLL/lymphoma 2	3, 48
	BIRC5	baculoviral IAP repeat-containing 5	36
	BSG	basigin (Ok blood group)	41
	CAV2	caveolin 2	5
	CCNA2	cyclin A2	43
	COL18A1	collagen, type XVIII, alpha 1	47
	DCN	decorin	28
	EPS15	epidermal growth factor receptor pathway substrate 15	28
	ERRFI1	ERBB receptor feedback inhibitor 1	45
	EZR	ezrin	18
	FBN1	fibrillin 1	36
	IRS2	insulin receptor substrate 2	6, 37
	ITGA6	integrin, alpha 6	30
	LRP5	low density lipoprotein receptor-related protein 5	6
	MARCKS (includes EG:4082)	myristoylated alanine-rich protein kinase C substrate	47
	SDC2	syndecan 2	8
	TAL1	T-cell acute lymphocytic leukemia 1	18
	THBS2	thrombospondin 2	11
	TIMP2	TIMP metallopeptidase inhibitor 2	11
	TMSB10	thymosin beta 10	19
	TRAF2	TNF receptor-associated factor 2	26
	VIM	vimentin	19, 36

To further analyze the possible role of these differentially expressed miRNAs in endometriosis, we performed a different analysis uploading the miRNAs directly in IPA. In this way, the software identified 6 networks, 3 of which are highly significant with known biological functions including genetic disorders, connective tissue disorders, skeletal and muscular disorders, cancer, and reproductive system disorders (Table 4).

The difference in the number of networks identified by IPA is ascribable to the different database used by the software, as IPA uses the Argonaute 2 databases (http://www.ma.uni-heidelberg.de/apps/zmf/argonaute/) to analyse miRNAs and their known or predicted targets, and this database identified only 118 targets for the 50 miRNAs.

Next, we performed an IPA analysis on the 1203 predicted targets of the miRNAs whose differential expression between eutopic and ectopic tissue was confirmed by real-time RT-PCR. IPA software identified 49 networks and revealed that the predicted targets were enriched for biological functions such as cellular development, cell morphology, cell-mediated immune response, gene expression, cell cycle, cell death, cancer, and developmental disorders. The network with the highest score from this analysis, shown in Figure 3, includes molecules that have been implicated in endometriosis such as the TNF receptor, IL10, IL6, and FOXO1 [49–55].

Performing the analysis uploading directly the miRNAs in IPA, thus using the Argonaute2 database, the software identified only one network (Figure 4), the major biological

Table 4: Molecular networks constituted by the predicted miRNA targets. The list of differentially expressed miRNAs was directly uploaded in IPA and an analysis was performed in order to identify the molecular pathways and functions to which the predicted targets of the differentially expressed miRNAs belong. The database used by IPA to analyze miRNAs and their targets is Argonaute2 (http://www.ma.uni-heidelberg.de/apps/zmf/argonaute/). $P < .01$.

ID	Molecules in Network	*P*-value	Focus Molecules	Top Functions
1	AKAP3, ATP2A2, C11ORF87, CNKSR2, **CREB1**, CUGBP2, EIF4E3, ELK1, FLRT2, HOXB2, HOXD12, IFNG, KLHDC10, KPNB1, MIR25, MIR150, MIR186, MIR221, MIR299, MIR143 (includes EG:406935), MIR182 (includes EG:406958), MIR200A, MIR200B, MIR200C, MIR34A, MYST4, OTOF, PAQR3, PER1, RPGRIP1L, SNRPA, SRCAP, UBFD1, USP6NL, WDR44	10E−24	11	Genetic Disorder, Skeletal and Muscular Disorders, Connective Tissue Disorders
2	**ATP1B1**, C4ORF16, CALU, DHX15, DIP2C, **DNMT3A**, **DNMT3B**, EVX2, FAM108C1, FBXL11, HOXA5, HOXA10, INO80, JPH3, KLHL18, MACF1, MAP2K6, MIR126, MIR100 (includes EG:406892), MIR130A (includes EG:406919), MIR130B (includes EG:406920), MIR132 (includes EG:406921), MIR148A (includes EG:406940), MIR20A, MIR29B, MIR29B1, MIR29B2, MIR29C, **MPPED2** (includes EG:744), NUFIP2, SMARCE1, SOX6, ZFP36L2, ZNF238, ZNF318 (includes EG:24149)	10E–19	9	Genetic Disorder, Skeletal and Muscular Disorders, Infection Mechanism
3	ADIPOR2, **AR**, ARF4, CAND1, CCNT2, CDKN1A, CHSY1, FBXW7, FNDC3B, IRS1, **JUN**, KLF6, LASS2, MAP1D, MDM2, MIR93, MIR375, MIR1 (human), MIR106A (includes EG:406899), MIR106B (includes EG:406900), MIR145 (includes EG:406937), MIR183 (includes EG:406959), MIR196B, MIR99A, MTPN, NPAT, NPPC, PDCD4, PFTK1, PPM1D, SERP1, SERPINB5, SLC16A2, TDG, TRIM2	10E−18	9	Cancer, Reproductive System Disease, Cell Cycle
4	MIR376A, MIR376A1, MIR376A2	10E−2	1	Genetic Disorder, Skeletal and Muscular Disorders
5	MIR365, MIR365-1, MIR365-2	10E−2	1	
6	EZH2, MIR101, MIR101-1, MIR101-2, MYCN	10E−2	1	Cancer, Cellular Movement, Reproductive System Disease

functions of which are cell cycle, cell death, and connective tissue disorders. This network contains PIK3R1, and its expression has been demonstrated to be upregulated in endometriosis, were it can play an essential role in TNF-mediated antiapoptotic signaling [56]. Another interesting molecule present in this pathway is SIP1, a validated target of the miR-200 family, which is a factor implicated in epithelial to mesenchymal transition and tumor metastasis [57]. Thus, the observed downregulation of miR-200 family in the ectopic endometrium may have a role in the endometrial lesion development.

We further investigated the function of the predicted targets of the RT-PCR-validated miRNAs by using Onto-Express and Pathway-Express (http://vortex.cs.wayne.edu/) in order to categorize the targets according to Gene Ontology (GO) and KEGG pathways, respectively [58, 59]. The predicted targets of the validated miRNAs were uploaded in Onto-Express and the list of the putative targets of the 475 miRNAs assayed was used as reference. Onto-Express calculates the mRNA targets in each GO category and compares it with the expected number of targets present in the GO category. Significant differences from

Table 5: Gene Ontology analysis of the predicted target genes of 50 miRNAs differentially expressed. Onto-Express analysis on predicted targets of the differentially expressed miRNAs identified enrichment for biological process categories. The *gene* column indicates the number of predicted targets of the differentially expressed miRNAs upon the number of the targets of all miRNAs considered for the study. Significant differences from the number of targets in each GO category with the expected number of genes were calculated with the assumption of a hypergeometric distribution and *P*-values were adjusted with the false discovery rate (fdr) correction. $P < .05$.

Rank	Biological process category	Genes	Corrected *P*-value
1	Cellular process	2408/6644	.0
	Cell motion	148/330	.0
	Cell communication	908/2223	.0
	Cellular component organization	546/1383	.0
	Cellular developmental process	400/949	.0
	Cellular metabolic process	1563/4269	.0
	Regulation of cellular process	1555/3840	.0
	Cell development	183/419	.0
	Positive regulation of cellular process	371/875	.0
	Negative regulation of cellular process	394/932	.0
	Cell cycle	223/555	1.0E−5
	Cell death	235/587	2.0E−5
	Cell proliferation	237/602	5.0E−5
	Actin-filament based process	90/197	6.0E−5
	Cell fate commitment	45/83	9.0E−5
	Cell aging	14/21	7.0E−4
	Vescicle-mediated transport	158/397	7.3E−4
	Cell growth	51/112	.00286
	Cell fate determination	15/23	.00286
	Cellular localization	224/609	.00506
	Gene silencing	16/27	.00696
	Cell cycle process	124/323	.00696
	Translational initiation	23/48	.01253
	Cell fate specification	12/20	.01728
	Cellular response to stimulus	110/292	.03290
	Cell adhesion	173/479	.04094
2	Negative regulation of biological process	421/992	.0
	Negative regulation of metabolic process	193/422	.0
	Negative regulation to cellular process	394/932	.0
	Negative regulation of developmental process	129/309	1.9E−4
	Negative regulation of response to stimulus	16/29	.01705
	Negative regulation of growth	24/53	.03564
3	Multicellular organismal process	820/2037	.0
	Multicellular organismal development	675/1606	.0
	Regulation of multicellular organismal process	171/421	2.0E−4
	System process	227/606	.00750
	Respiratory gaseous exchange	11/17	.01639
4	Biological regulation	1656/4148	.0
	Regulation of molecular function	211/478	.0
	Regulation of biological process	1597/3961	.0
	Regulation of biological quality	281/732	1.3E−4

Table 5: Continued.

Rank	Biological process category	Genes	Corrected *P*-value
5	Regulation of biological process	1597/3961	.0
	Regulation of metabolic process	850/2038	.0
	Regulation of developmental process	283/657	.0
	Regulation of cellular process	1555/3840	.0
	Positive regulation of cellular process	384/933	.0
	Negative regulation of cellular process	421/992	.0
	Regulation of multicellular organismal process	171/421	1.4E−4
	Regulation of localization	110/261	8.7E−4
	Regulation of locomotion	41/95	.02619
	Regulation of growth	64/164	.04199
6	Metabolic process	1631/4509	.0
	Biosynthetic process	899/2354	.0
	Negative regulation of metabolic process	193/422	.0
	Positive regulation of metabolic process	201/473	.0
	Regulation of metabolic process	1563/2038	.0
	Cellular metabolic process	1563/4269	.0
	Primary metabolic process	1551/4187	.0
	Macromolecule metabolic process	1383/3644	.0
	Oxydation reduction	52/255	5.0E−5
	Catabolic process	237/665	.01317
	Nitrogen compound metabolic process	49/193	.03270
7	Developmental process	821/1967	.0
	Multicellular organismal development	675/1606	.0
	Anatomical structure morphogenesis	310/710	.0
	Embryonic development	140/304	.0
	Anatomical structure development	584/1379	.0
	Cellular developmental process	400/949	.0
	Regulation of developmental process	283/657	.0
	Positive regulation of developmental process	131/295	1.0E−5
	Anatomical structure formation involved in Morphogenesis	97/216	4.0E−5
	Pattern specification process	79/173	1.6E−4
	Negative regulation of developmental process	129/309	1.6E−4
	Pigmentation during development	9/13	.01264
	Reproductive developmental process	31/68	.02708
	Aging	17/36	.04082
8	Positive regulation of biological process	384/933	.0
	Positive regulation of metabolic process	201/473	.0
	Positive regulation of cellular process	371/875	.0
	Positive regulation of developmental process	131/295	1.0E−5
	Positive regulation of homeostatic process	6/8	.03203
9	Localization	715/1953	.0
	Localization of cell	148/330	.0
	Macromolecule localization	247/638	1.1E−4
	Regulation of localization	110/261	7.7E−4
	Cellular localization	224/609	.00422
	Establishment of localization	577/1657	.00463

Table 5: Continued.

Rank	Biological process category		Genes	Corrected *P*-value
10	Death		235/591	2.0E−5
	Cell death		235/587	1.0E−5
11	Anatomical structure formation		242/629	1.1E−4
	Anatomical structure formation involved in	Morphogenesis	97/216	3.0E−5
	Cellular component assembly		165/452	.01276
12	Response to stimulus		464/1276	2.4E−4
	Response to chemical stimulus		185/465	5.3E−4
	Response to endogenous stimulus		59/136	.00633
	Negative regulation to response to stimulus		16/29	.01844
	Behavior		84/215	.02638
	Cellular response to stimulus		110/292	.03534
	Response to stress		253/718	.03918
13	Multi-organism process		113/286	.00251
	Interspecies interaction between organisms		71/172	.00565
	Female pregnancy		19/39	.04504
14	Growth		96/235	.00334
	Cell growth		51/112	.00298
	Negative regulation of growth		24/53	.03391
	Regulation of growth		64/164	.03916
15	Locomotion		111/277	.00422
	Cell motility		97/223	3.5E−4
	Regulation of locomotion		41/95	.02439
16	Establishment of localization		577/1657	.00458
	Establishment of protein localization		207/536	3.3E−4
	Establishment of localization in cell		209/576	.01045
17	Reproduction		117/303	.00983
	Reproductive process		116/301	.01127
18	Reproductive process		116/301	.01024
	Reproductive developmental process		31/68	.03090
	Female pregnancy		19/39	.04504
19	Biological adhesion		173/479	.03486
	Cell adhesion		173/479	.03486
20	Rhythmic process		26/59	.04158

the expected number of genes were calculated assuming a hypergeometric distribution, and P values were adjusted with the false discovery rate correction based on the number of GO categories tested. A corrected P value $<$ .05 was considered statistically significant. Onto-Express analysis revealed enrichment for several biological processes known to be relevant in endometriosis, such as developmental process, cell death, cell cycle, and cell adhesion (Table 5).

Pathway-Express analysis identified 33 pathways significant at 5% level (Table 6), most of which are coherent with the current knowledge on endometriosis. For instance, the most significant pathways putatively affected by the differential expression of miRNAs are *MAPK* and *axon guidance* the latter shown in Figure 5. While *MAPK* pathway, which is involved in several cellular functions, such as cell proliferation, migration, and differentiation, is clearly relevant for endometriosis, *axon guidance*, at first may appear unrelated to this pathology. However, nerves and blood vessels are highly interconnected, both physically and in their morphogenesis. Indeed, it has been demonstrated that several molecules involved in axon guidance, such as semaphorins, plexins, and neuropilins, are also strongly implicated in angiogenesis [60], a biological process essential for endometriosis. Intriguingly, this pathway contains ROBO1, and its expression, higher in ectopic endometrium compared to eutopic tissue, positively correlates with endometriosis recurrence [61], thus suggesting that miRNAs may take part in tuning ROBO1 expression and have a role in the recurrence of the pathology.

Table 6: KEGG pathways containing the predicted targets of the differentially expressed miRNAs. Pathway-Express analysis identified the KEGG molecular pathways affected by the predicted targets of the differentially expressed miRNAs. $P < .05$.

Rank	Pathway name	Genes in pathway	Input genes in pathway	Pathway genes on chip	*P*-value
1	MAPK signaling pathway	272	103	197	3.23E−08
2	Axon guidance	129	67	113	3.23E−08
3	Melanogenesis	102	48	74	8.60E−08
4	Pathways in cancer	330	119	245	2.27E−07
5	Regulation of actin cytoskeleton	217	78	158	2.31E−05
6	Focal adhesion	203	75	150	2.31E−05
7	Wnt signaling pathway	152	63	127	1.60E−04
8	Glioma	65	30	50	2.94E−04
9	GnRH signaling pathway	103	36	65	4.86E−04
10	Renal cell carcinoma	69	34	61	5.92E−04
11	Insulin signaling pathway	138	49	98	7.02E−04
12	Adherens junction	78	34	62	7.65E−04
13	TGF-beta signaling pathway	87	38	72	8.49E−04
14	Prostate cancer	90	36	68	.0011
15	ECM-receptor interaction	84	30	55	.0016
16	Phosphatidylinositol signaling system	76	30	55	.0016
17	Calcium signaling pathway	182	54	115	.0016
18	Colorectal cancer	84	36	70	.0018
19	Long-term potentiation	73	31	58	.0018
20	Adipocytokine signaling pathway	67	27	50	.0032
21	ErbB signaling pathway	87	34	69	.0056
22	Pancreatic cancer	72	30	59	.0056
23	Gap junction	96	33	67	.0063
24	Type II diabetes mellitus	45	18	31	.0069
25	Small cell lung cancer	86	30	61	.0095
26	Thyroid cancer	29	14	23	.0111
27	Ubiquitin mediated proteolysis	138	42	94	.0145
28	Long-term depression	75	24	49	.0225
29	Non-small cell lung cancer	54	20	39	.0225
30	Acute myeloid leukemia	59	22	45	.0304
31	Melanoma	71	25	53	.0323
32	Cardiac muscle contraction	87	20	41	.0402
33	Chronic myeloid leukemia	75	28	62	.0410

3.4. Genes Differentially Expressed in Endometriosis Are Predicted Targets of the Differentially Expressed miRNAs. Finally, after the identification of the predicted targets of the differentially expressed miRNAs, we investigated whether they were in accordance with the results of two studies of gene expression in endometriosis. We first analysed the genes reported to be differentially expressed in a study on paired eutopic and ectopic samples of ovarian endometriosis [23]. This study identified 701 differentially expressed transcripts (expression ≥ 0.2; fold change $\pm \geq 2$; $P \leq .05$), 82 of which are predicted target genes of the 50 miRNAs, 51/492 upregulated and 31/209 downregulated. A second study on peritoneal endometriosis [24] identified 622 differentially expressed transcripts (fold change $\pm \geq 1.5$; $P \leq .05$), 107 of which are predicted targets of the differentially expressed miRNAs, 73/232 upregulated and 34/390 downregulated. Hypothesising that the genes differentially expressed common to both studies are likely those specific to endometriosis independently from the site of the lesion, we restricted the analysis to the differentially regulated genes in eutopic and ectopic endometrium common to the two studies that are also predicted targets of the 50 miRNAs (Table 7). IPA analysis identified 5 molecular networks, the most relevant functions of which being cancer, cell cycle, and reproductive system disease (Table 8). The overlap of networks generated by IPA is shown in Figure 6. In this graphical representation the most relevant nodes are the transcription factor SP1, tumor necrosis factor (TNF), and SRC, in remarkable agreement with the nodes of the most significant networks obtained by IPA analysis performed on the distinct datasets of differentially expressed genes in ovarian and peritoneal endometriosis (data not shown).

Table 7: Genes aberrantly expressed in ovarian and peritoneal endometriosis that are predicted targets of the differentially expressed miRNAs. The miRNAs predicted to regulate the expression of the genes known to be aberrantly up- (↑) or downregulated (↓) in both ovarian and peritoneal endometriosis were identified by TARGETSCAN and PICTAR algorithms. MicroRNAs whose regulation is in accordance with the resulting expression of their predicted target genes are reported in bold.

Target genes	microRNAs upregulated	microRNAs downregulated
CA3 (carbonic anhydrase III) ↑	hsa-miR-29b; hsa-miR-29c	
CAV1 (caveolin 1) ↑	hsa-miR-199a; hsa-miR-30e-3p	**hsa-miR-20a; hsa-miR-106b**
CAV2 (caveolin 2) ↑	hsa-miR-29b; hsa-miR-29c	
DMD (dystrophin) ↑	hsa-miR-101; hsa-miR-30e-5p	**hsa-miR-200b; hsa-miR-200c**
EPHA3 (EPH receptor A3) ↑	hsa-miR-29b; hsa-miR-29c	**hsa-miR-182**
FZD7 ↑ (frizzled homolog 7)	hsa-miR-145; hsa-miR-1	**hsa-miR-20a; hsa-miR-106b**
GALNT3 (UDP-N-acetyl-alpha-D-galactosamine) ↓	**hsa-miR-30e-5p**	
KCNMA1 (potassium large conductance calcium-activated channel, subfamily M, alpha mamber 1) ↑	hsa-miR-186	**hsa-miR-93; hsa-miR-17-5p; hsa-miR-20a; hsa-miR-106b**
LMO3 (LIM domain only 3) ↑		**hsa-miR-20a; hsa-miR-93; hsa-miR-17-5p; hsa-miR-183; hsa-miR-106b**
NFASC (neurofascin) ↑	hsa-miR-150	**hsa-miR-200b; hsa-miR-200c; hsa-miR-182**
PDE4DIP (phosphodiesterase 4D interacting protein) ↑		**hsa-miR-183**
PLS1 (plastin 1) ↓	**hsa-miR-30e-5p**	hsa-miR-17-5p; hsa-miR-20a; hsa-miR-106b
PTPN3 (protein tyrosine phosphatase, non-receptor type 3) ↓		hsa-miR-17-5p; hsa-miR-20a; hsa-miR-106b
RGS2 (regulator of G-protein signalling 2) ↑	hsa-miR-30e-5p	**hsa-miR-182**
RGS5 (regulator of G-protein signalling 5) ↑	hsa-miR-186	
RPS6KA5 (ribosomal protein S6 kinase, 90 kDa, polypeptide 5) ↓	**hsa-miR-148a**	hsa-miR-93; hsa-miR-17-5p; hsa-miR-20a; hsa-miR-106b
SCAP2 (src family associated phosphoprotein 2) ↑		**hsa-miR-182**
SLCO3A1 (solute carrier organic anion transporter family, member 3A1) ↑	hsa-miR-34a	**hsa-miR-182**
SNAP25 (synaptosomal-associated protein) ↑	hsa-miR-130a; hsa-miR-1	**hsa-miR-130b; hsa-miR-200b; hsa-miR-200c**
TNFSF12 (tumor necrosis factor superfamily, member 12) ↑	hsa-miR-28	

4. Conclusions

MicroRNAs are predicted to regulate a large fraction of protein-coding genes, as computational analysis reveals that an average miRNA could have as many as 100 or more target genes. On the other hand, a single gene may have target sites for several distinct miRNAs, allowing a fine tuning of gene expression by miRNAs.

In the present study, we used miRNA microarray technology to identify the miRNAs differentially expressed in paired eutopic/ectopic endometrium from the same patients and bioinformatics tools to identify their predicted targets as well as the molecular networks and the biological functions they may affect.

Comparing miRNA expression profiles among the different subjects, we identified 50 miRNAs differentially expressed in ectopic *versus* eutopic samples. Several of these miRNAs were also reported to be differentially expressed in two recent studies [62, 63], although with a modulation occasionally discordant from our results. This, joint to a notable accordance between their predicted targets and the genes reported to be differentially expressed in two studies of gene expression [23, 24], consolidates the hypothesis of a possible role of miRNAs in the pathogenesis of endometriosis.

The miRNAs-predicted targets were identified by the intersection of the results from two different search algorithms, and the biological functions the differentially expressed miRNA may affect were identified by Onto-Express and IPA software. Functional analysis, performed using IPA software, was carried out uploading either the predicted targets or the differentially expressed miRNAs, thus using different databases for miRNA targets. As expected,

Table 8: Molecular networks constituted by the common differentially expressed transcripts in ovarian and peritoneal endometriosis predicted to be targets of the 50 miRNAs. Differentially expressed genes common to both ovarian and peritoneal endometriosis that are predicted targets of the 50 differentially expressed miRNAs were uploaded in IPA in order to identify the molecular networks and functions to which they belong. $P < .01$.

ID	Molecules in Network	*P*-value	Focus Molecules	Top Functions
1	CAV1, CAV2, CDKN1A, ESR1, HMGA1, LPL, MMP2, NOS3, SMARCA4, SP1, SP3, SRC, TNFSF12, TP53	10E−8	4	Cancer, Cell Cycle, Reproductive System Disease
2	MBD1, SLCO3A1	10E−2	1	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
3	RGS2, TNF	10E−2	1	Lipid Metabolism, Small Molecule Biochemistry, Cell Signaling
4	DMD, DTNA, DTNB	10E−2	1	Cellular Assembly and Organization, Nervous System Development and Function, Skeletal and Muscular System Development and Function
5	FYB, GRB2, SKAP2	10E−2	1	Cell-To-Cell Signaling and Interaction, Cell-mediated Immune Response, Cellular Growth and Proliferation

the different algorithms used to predict miRNA targets led to the identification of different molecular networks. Still, in both cases, the identified networks contained several transcripts known to be implicated in endometriosis and with their main biological functions linked to the disease. Since the targets of miRNAs are just predictions based on mathematical algorithms, the choice of the algorithm may radically modify on the whole the list of the predicted target genes and that of the molecular networks they belong to. For this reason, the validation of miRNA targets in vitro, in a cellular system, is essential to evaluate the contribution of each miRNA to the overall modulation of gene expression.

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