MicroRNA-Regulated Pathways Associated with Endometriosis

E. Maria C. Ohlsson Teague, Kylie H. Van der Hoek, Mark B. Van der Hoek, Naomi Perry, Prabhath Wagaarachchi, Sarah A. Robertson, Cristin G. Print, and M. Louise Hull

Research Centre for Reproductive Health (E.M.C.O.T., K.H.V., N.P., S.A.R., M.L.H.), School of Paediatrics and Reproductive Health, University of Adelaide, and Adelaide Microarray Centre (M.B.V.), Hanson Institute, Adelaide, South Australia 5005, Australia; Department of Gynaecology (P.W.), Women’s and Children’s Hospital, Adelaide, South Australia 5006, Australia; and Department of Molecular Medicine and Pathology (C.G.P.), University of Auckland, Private Bag 92019, Auckland, New Zealand

Endometriosis is a prevalent gynecological disease characterized by growth of endometriotic tissue outside the uterine cavity. MicroRNAs (miRNAs) are naturally occurring posttranscriptional regulatory molecules that potentially play a role in endometriotic lesion development. We assessed miRNA expression by microarray analysis in paired ectopic and eutopic endometrial tissues and identified 14 up-regulated (miR-145, miR-143, miR-99a, miR-99b, miR-126, miR-100, miR-125b, miR-150, miR-125a, miR-223, miR-365, miR-29c and miR-1) and eight down-regulated (miR-200a, miR-141, miR-200b, miR-142-3p, miR-424, miR-34c, miR-20a and miR-196b) miRNAs. The differential expression of six miRNAs was confirmed by quantitative RT-PCR. An in silico analysis identified 3851 mRNA transcripts as putative targets of the 22 miRNAs. Of these predicted targets, 673 were also differentially expressed in ectopic vs. eutopic endometrial tissue, as determined by microarray. Functional analysis suggested that the 673 miRNA targets constitute molecular pathways previously associated with endometriosis, including c-Jun, CREB-binding protein, protein kinase B (Akt), and cyclin D1 (CCND1) signaling. These pathways appeared to be regulated both transcriptionally as well as by miRNAs at posttranscriptional level. These data are a rich and novel resource for endometriosis and miRNA research and suggest that the 22 miRNAs and their cognate mRNA target sequences constitute pathways that promote endometriosis. Accordingly, miRNAs are potential therapeutic targets for treating this disease. (Molecular Endocrinology 23: 265–275, 2009)
microRNAs (miRNAs) are highly conserved, 20- to 24-nucleotide single-stranded RNAs that bind to single or multiple 7- to 8-mer motifs situated predominantly, but not exclusively, in the 3'-untranslated regions of target mRNAs. In mammalian cells, this generally leads to mRNA destabilization and/or translational repression, depending on the degree of sequence homology between the miRNA and target RNA transcript. Two recent proteomic studies showed that a single miRNA might curtail the synthesis of proteins encoded by hundreds of genes (12, 13). There may be up to 1000 miRNAs (14) targeting approximately 30% of the human genome and thereby regulating transcripts from as many as 8000 genes (15).

miRNAs are potent negative feedback regulators in a broad array of cellular processes that occur in endometriosis, such as cell differentiation and proliferation (16–19), cell migration (20–23), and myogenesis (16, 19, 24). Aberrant miRNA expression has been associated with complex disorders such as hematopoietic disease (18), heart failure (25), and several cancers including estrogen-regulated ovarian (26) and breast cancer (27). Therapeutic antagonism of miRNAs has been successful in nonhuman primates (28) and may therefore become a novel foundation for future research into the role of miRNA-associated human disease.

We hypothesized that miRNAs mediate posttranscriptional regulation in endometriotic lesions and regulate transcripts in previously identified endometriosis-associated molecular networks (10). Therefore, miRNA expression in paired eutopic and ectopic endometrial biopsies from patients with endometriosis was compared. Using microarray, quantitative RT-PCR (qRT-PCR), and bioinformatic techniques, we have identified both novel and previously characterized miRNA-regulated molecular pathways that are likely to contribute to the pathogenesis of endometriosis. These findings constitute a comprehensive foundation for future research into the role of miRNAs in this disease.

**Results**

**miRNAs are differentially expressed in ectopic endometrial tissue**

To determine the miRNA profile in endometrial tissue, miRNA microarray analysis was performed on sets of paired samples of eutopic and peritoneal ectopic endometrial tissue from seven patients with endometriosis. Three distinct computational analyses, Independent Component Analysis (ICA), supplemental Fig. 1A published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org), ANOVA, and Linear models for microarray data (Limma) (30) all identified 22 miRNAs that were differentially expressed with a fold change $\pm 1.5$ at $P \leq 0.05$ in eutopic vs. ectopic endometrium (supplemental Fig. 1B). Fourteen miRNAs were up-regulated, and eight were down-regulated (Table 1) in ectopic tissue.

An ANOVA analysis on the miRNA microarray data according to menstrual cycle phase was also performed using Limma. This failed to show any significant differences in miRNA profiles between tissues taken from women during proliferative ($n = 4$) and secretory ($n = 3$) phases (data not shown).

**qRT-PCR analysis of endometrial miRNA expression**

To validate the microarray methods, three up-regulated (miR-99a, miR-126, miR-145) and three down-regulated (miR-141, miR-200b, and miR-424) miRNAs were quantified using qRT-PCR analysis (Fig. 1). In concordance with the microarray, qRT-PCR showed significant differential expression of these miRNAs in ectopic vs. eutopic endometrium ($P \leq 0.05$). miR-145 and miR-141 were the most differentially expressed of the six miRNAs ($P \leq 0.001$), up- and down-regulated, respectively. The qRT-PCR $\Delta Ct$ values were normalized to miR-let-7a and miR-let-7d, which were both highly abundant and invariably expressed (fold change $= 1.09$ and 1.05, respectively; $P = 0.5$) according to the microarrays.

**miRNA gene families and clusters**

The mean miRNA fold changes, chromosomal locations, family memberships and cluster affiliations for the 22 differentially expressed miRNAs are presented in Table 1. These include both inter- and intragenic miRNAs. Two up-regulated (miR-125 and miR-99) and one down-regulated (miR-8) miRNA gene families, and three clusters (defined as groups of miRNAs located within 10 kb of each other) were identified among the differentially regulated miRNAs.

**Identification of miRNA targets in endometriosis**

The predicted target miRNAs of the 22 differentially expressed miRNAs were identified in silico. A conservative approach was undertaken, limiting the targets to those predicted by two algorithms, TargetScanS (31) and four-way PicTar (32), hereafter referred to as the two-way intersection. Although no targets were listed for miR-365 and miR-424 in the four-way PicTar algorithm, 3851 target transcripts corresponding to 2340 unique official gene symbols were present in this intersection for the remaining 20 miRNAs (supplemental Table 1 published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org). The number of miRNA targets predicted for each of the differentially expressed miRNAs varied substantially (Fig. 2A).

**Functional analysis of miRNA targets in endometriosis**

The functions of the 3851 predicted miRNA target transcripts and the molecular pathways they potentially constitute were assessed using Ingenuity Pathway Analysis (IPA) software. The predicted targets were significantly enriched for several bi-
ological functions known to be involved in endometriosis, including: cellular movement, assembly and organization; cell-to-cell signaling and interaction; nervous system development and function; embryogenesis; cell cycle; cell death; connective tissue development and function and endocrine system development and function (supplemental Table 2).

IPA identified 39 molecular networks constituted by the 3851 predicted targets, exemplified by two networks with well-characterized miRNA targets in Fig. 2.

The network in Fig. 2B includes myocyte enhancer factor 2C (MEF2C), a confirmed target (18) of one of the up-regulated miRNAs in ectopic endometrium in this study (miR-223). IPA suggested that 22 of the 3851 predicted targets of the differentially expressed miRNAs had molecular associations with MEF2C in the published literature (supplemental Fig. 2A). It is possible that groups of molecular associations such as these occur by chance. Therefore, as proof of principle, a permutation study was performed. This showed that only 4.4% of 1000 randomly selected lists of 22 miRNAs had more predicted targets associated with MEF2C in the IPA database than the 22 differentially expressed miRNAs (supplemental Fig. 2B). A corresponding permutation analysis focused on c-Jun, another target of interest in our study, showed similar results (supplemental Fig. 2, C and D).

Another molecular network constituted by the 3851 predicted targets converged on interferon-γ/H9253 and included TGFβ/H9252, secreted protein, acidic, cysteine-rich, osteonectin (SPARC), and collagens COL1A1 and COL1A2, which are all confirmed targets of the ectopically dysregulated miR-141 (33), and miR-29c (23), respectively. Mechanisms mediated by these molecules have been implicated in endometrial lesion development (10, 34), and the network in Fig. 2C suggests that these pathways may also be regulated by miRNAs.

| Human (hsa-) miRNA gene | Fold Change Accession no. Chr Chromosomal location miRNA family miRNA cluster |
|------------------------|---------------------|-----------------|-----------------|-----------------|-----------------|
| miR-145                | 4.47                | MIMAT0000437 5  | Intergenic      | mir-145         | 1               |
| miR-143                | 2.84                | MIMAT0000435 5  | Intergenic      | mir-143         | 1               |
| miR-99a                | 2.46                | MIMAT0000097 21 | Overlapping transcripts | mir-99         | 2               |
| miR-99b                | 2.43                | MIMAT0000689 19 | Intergenic      | mir-99         | 2               |
| miR-126                | 2.33                | MIMAT0000445 9  | Overlapping transcripts | mir-126      | 2               |
| miR-100                | 2.25                | MIMAT0000098 11 | Intergenic      | mir-99         | 2               |
| miR-125b               | 1.96                | MIMAT0000432 11,21 | Intergenic | mir-125        | 2               |
| miR-150                | 1.95                | MIMAT0000451 19 | Overlapping transcripts | mir-150      | 2               |
| miR-125a               | 1.79                | MIMAT0000443 19 | Intergenic      | mir-125        | 2               |
| miR-223                | 1.72                | MIMAT0000681 X  | Intergenic      | mir-223        | 2               |
| miR-194                | 1.67                | MIMAT0000460 1,11 | Overlapping transcripts | mir-194      | 2               |
| miR-365                | 1.65                | MIMAT0000710 16,17 | Overlapping transcripts | mir-365      | 2               |
| miR-29c                | 1.63                | MIMAT0000682 1  | Intergenic      | mir-29         | 2               |
| miR-1                 | 1.53                | MIMAT0000416 20,18 | Overlapping transcripts | mir-1       | 2               |
| miR-196b               | −1.60               | MIMAT0000180 7  | Overlapping transcripts | mir-196      | 2               |
| miR-20a                | −1.80               | MIMAT0000075 13 | Overlapping transcripts | mir-17       | 2               |
| miR-34c                | −1.91               | MIMAT0000686 11 | Intergenic      | mir-34         | 2               |
| miR-424                | −2.27               | MIMAT0001341 X  | Overlapping transcripts | mir-322      | 2               |
| miR-142–3p             | −2.27               | MIMAT0000434 17 | Intergenic      | mir-142       | 2               |
| miR-200b               | −2.28               | MIMAT0000318 1  | Intergenic      | mir-8          | 3               |
| miR-141               | −2.34               | MIMAT0000432 12 | Intergenic      | mir-8          | 3               |
| miR-200a               | −2.67               | MIMAT0000682 1  | Intergenic      | mir-8          | 3               |

miRNA genes significantly (P ≤ 0.05) differentially regulated (fold change ≥ 1.5) determined by ANOVA from microarray analysis of ectopic compared with eutopic endometrial tissue from seven patients with endometriosis are listed in order of their fold change. The accession number is for the mature miRNA sequence; Chr, Chromosome.

**FIG. 1.** qRT-PCR analysis of miRNA expression in endometriosis. miRNA expression in paired ectopic and eutopic endometrial samples from women in endometriosis, as determined by microarray (n = 7, black) and qRT-PCR (n = 8). In qRT-PCR analysis, miRNA expression was normalized to hsa-miR-let-7a (dark gray) or hsa-miR-let-7d (light gray). Significant fold changes in ectopic vs. eutopic endometrium are marked by *, P ≤ 0.05; **, P ≤ 0.01; or ***, P ≤ 0.001. Data are mean ± SEM.

**TABLE 1.** Differentially expressed miRNAs in paired ectopic vs. eutopic endometrial tissue from patients with endometriosis

Mol Endocrinol, February 2009, 23(2):265–275
Gene Ontology (GO) analysis suggested that the predicted targets of the 22 differentially expressed miRNAs were relatively enriched for GO paths associated with transcription. However, targets of five randomly chosen sets of 22 miRNAs were equally enriched for this category of GO paths (data not shown).

mRNAs differentially expressed in endometriosis are predicted targets of the 22 endometrial miRNAs

After identification of the 22 differentially expressed miRNAs and their predicted targets, we further restricted our analysis to a subset of putative miRNA targets that were also differentially expressed at the mRNA level (summarized in supplemental Fig. 3). In an mRNA microarray study of paired eutopic and peritoneal ectopic endometrium from women with endometriosis (10), hereafter referred to as the peritonal comparison, 4034 transcripts were differentially expressed (fold change $\geq 1.5$, $P \leq 0.05$). Of these transcripts, 673 (572 unique official gene symbols) were both differentially expressed and predicted to be targeted by at least one, but often several, of the 22 differentially expressed miRNAs (supplemental Table 3). IPA suggested that the 673 transcripts were significantly enriched for functional annotations associated with endometriosis, including: cell death; connective tissue, nervous and muscular system development and function; cellular movement; cell proliferation and the cell cycle; angiogenesis and reproductive/endocrine system disorders (supplemental Table 4).
FIG. 3. Functional analysis of differentially expressed miRNA targets in endometriosis. A–C, Networks identified by IPA of differentially expressed miRNAs that are also predicted targets of the 22 differentially expressed miRNAs in paired peritoneal ectopic vs. eutopic endometrial tissues from women with endometriosis. A, c-Jun is central in a network comprising extracellular matrix proteins that are confirmed targets of miRNAs differentially expressed in endometriosis. This network overlapped considerably to analysis of all predicted targets of the 22 miRNAs (Fig. 2C), suggesting a role for miRNAs in regulating cell migration during endometrial lesion development. B, MEF2C, histone deacetylase 4 (HDAC4), and DNA (cytosine-5-) methyltransferase 3α (DNMT3A) are validated miRNA targets represented in a network converging on CREBBP, proposing a role for miRNAs in the regulation of myogenesis, angiogenesis, and epigenetic modulation of gene expression in endometrial tissue remodeling. C, The IPA network converging on AKT indicated a role for miRNAs in regulating retinoic acid signaling, the cell cycle, and cellular growth and proliferation. D, IPA network from functional analysis of a second microarray data set of differentially expressed miRNA targets in paired ovarian ectopic vs. eutopic endometrial tissue from women with endometriosis. The network exemplifies the substantial overlap between the ovarian and the peritoneal comparisons, illustrated in orange and blue nodes. Red and green nodes depict up- and down-regulated miRNA targeted transcripts in ectopic vs. eutopic endometrial tissue, respectively. Lines represent the biological relationship between two nodes. The gene names in these networks may be identified on the HUGO Gene Nomenclature Committee home page (http://www.genenames.org).
These 673 mRNA transcripts were overrepresented in 12 IPA networks converging on 1) JUN; 2) platelet-derived growth factor β polypeptide/chemokine (C-X-C motif) ligand 12 (PDGF BB/CXCL12); 3) CREBBP; 4) CCND1; 5) calmodulin; 6) NFκB; 7) MAPK; 8) caveolin 1/vinculin (CAV1/VCL); 9) c-AMP dependent protein kinase (PKA); 10) phosphoinositide-3-kinase (PI3K); 11) protein kinase C (PKC); and 12) AKT (Fig. 3, A–C; supplemental Fig. 4, A–I; and supplemental Table 4). Although each of these was originally identified as a distinct network, there was cross-interaction between the networks, exemplified by JUN in Fig. 3A, which is a transcriptional target of MEF2C in Fig. 3B. Overlap was also found between networks of all predicted targets of the 22 miRNAs and networks of the differentially expressed predicted targets of the 22 miRNAs, illustrated by TGFβ2, SPARC, COL1A1, and COL1A2 in Figs. 2C and 3A.

To verify that the endometriosis-specific functions and networks identified here were not limited to the specific endometrial lesion types and patients examined, we also defined the intersection of miRNA data with a second mRNA microarray study of paired eutopic and ovarian ectopic endometriotic tissues (9), hereafter referred to as the ovarian comparison. This revealed 684 transcripts that were both differentially expressed (fold change ≥ ±1.5, P ≤ 0.05) and putatively targeted by one or more of the 22 differentially expressed miRNAs (supplemental Fig. 5 and supplemental Table 5).

The IPA networks identified for these 684 ovarian endometrial transcripts showed a remarkable overlap with those associated with the peritoneal comparison as exemplified in Fig. 3D, including those converging on AP1 (c-JUN/c-FOS), PI3K/AKT, CCND1, NFκB, and PDGF/BB (data not shown).

c-Jun mRNA expression

c-Jun is centrally located in one of the IPA networks (Fig. 3A) and is up-regulated at the mRNA level in the ectopic peritoneal endometrial tissue study (10). We used qRT-PCR to confirm the significant up-regulation of c-Jun mRNA (P = 0.015) in six of the same RNA samples used in the miRNA microarray and qRT-PCR analyses. Median normalized c-Jun expression in ectopic and eutopic tissue was 2.03 and 0.11, respectively (Fig. 4).

Discussion

This study used microarray technology, qRT-PCR, and in silico analyses in a novel strategy to assess miRNA expression in peritoneal endometrial tissues from women with endometriosis. Twenty-two miRNAs that were differentially expressed in paired ectopic vs. eutopic endometrium were identified that putatively regulate the expression of 2340 genes. A subset of these target transcripts was previously found to be differentially expressed between endometrial ectopic and eutopic endometrium in two separate mRNA microarray studies (9, 10). The specific expression profiles of the 22 miRNAs and their predicted targets may cause alterations to molecular pathways that are pivotal in the endometriotic process. Therefore, the inhibition or reactivation of these pathways by synthetic antisense antagomiRs or over expression of these miRNAs are potential therapeutic interventions in endometriosis.

Because ectopic endometrial tissue is less susceptible to steroid hormone-driven fluctuations than eutopic endometrium from women with endometriosis (reviewed in Ref. 35), miRNA analysis in different phases of the cycle would be expected to mainly reflect alterations in eutopic tissue. Therefore, miRNA expression was studied in endometrial tissues during both secretary (n = 3) and proliferative (n = 4) phases. The 22 differentially expressed miRNAs are hence consistently associated with endometriosis across the menstrual cycle. No effect on menstrual cycle phase on endometrial miRNA expression could be demonstrated in the present study. Cycle-related effects may be revealed by analysis of a larger study group in the future.

The dataset of 22 differentially regulated miRNAs in endometriosis was defined conservatively by the concordance of three methods of analysis. The combination of two parametric statistical methods (linear models that use the pooled correlation of replicate spots on the arrays, as well as a nested linear model design) with an independent nonparametric method (ICA) as well as qRT-PCR confirmation of three up- and three down-regulated miRNAs lends weight to the validity of this dataset.

Although all the 22 miRNAs have overlapping target lists, seven had the potential to individually target up to 494 mRNAs. Each of the 22 miRNAs was predicted to suppress, on average, 175 target mRNAs, with some miRNAs predicted to be coregulated by multiple miRNAs. This indicates that miRNAs, like transcription factors, may be considered master regulators in cellular processes in endometriosis.

proximally paired miRNA genes located up to 50 kb apart are frequently coexpressed (36). The finding of three miRNA clusters among the differentially expressed miRNAs provides further confidence in the results of this analysis. miRNAs in the same cluster have common predicted miRNA targets and might consequently have an additive repressive effect on target expression. The clustered and multitargeting miRNAs may be especially powerful regulators in ectopic endometrial lesions.

Approximately 80% of miRNA genes are located within introns (37), and these often have expression profiles that correlate with their host genes (36). We identified significant up-regulation in ectopic endometrial tissue of both miR-126
and epidermal growth factor (EGF)-like-domain, multiple 7 (EGFL7), a gene expressed in endothelial cells promoting vascular tubulogenesis. Because miR-126 is embedded in the EGFL7 gene, it is likely that these two genes are coexpressed in endometriosis, as previously described for miR-10a and homeobox B4 (38).

Correct identification of miRNA targets is central to determining the biological function of miRNAs. In this study, miRNA targets were identified at a conservative two-way intersection of TargetScanS and PicTar (four-way), as recommended in a recent consensus review (39). These are sensitive algorithms with substantial overlap in their predicted targets (40). miRNA targets with binding sites that were not conserved across species are excluded in this intersection, and we acknowledge that the impact of these miRNA targets in endometriosis was not explored here. However, in vitro studies suggested that this two-way intersection had a true positive rate of up to 90% (41) and that these two prediction models showed superior performance in a high-throughput examination of miRNA repression at protein level (12).

Frequent revision of miRNA target algorithms as well as target mRNA ENSEMBL annotations can dramatically alter predicted target transcript lists over time; therefore experimental confirmation of miRNA targets remains the gold standard. Despite the lack of large-scale screening methods for target verification, the number of validated miRNA targets is escalating, and there is growing evidence that miRNA targets can be confidently predicted in silico.

A large number of the miRNA targets of the 22 endometrial miRNAs discussed here are already experimentally confirmed in cellular systems similar to those involved in endometriosis. In addition, many of the molecular pathways these targets constitute are known to play important roles in the pathological processes involved in this condition. Therefore, we are confident that the molecular networks identified here represent conservative and well-founded hypotheses on which to base further experiments. Future studies are also necessary to determine the specific miRNA profiles of stromal and glandular epithelial cells as well as populations of infiltrating leukocytes, because the ratio of these cell types are altered in ectopic compared with eutopic endometrial tissue.

To appraise the general principle of identifying molecular networks using a systems biology database such as IPA, the associations with MEF2C and JUN of predicted targets of random miRNA datasets were assessed. These individual permutation studies suggested that, at least for these two genes, the results of the functional analysis were unlikely to occur by chance.

Functional analysis of miRNA targets was limited to the convergence of miRNA and mRNA microarray data. This method focuses the analysis not only on the potential actions of miRNAs in translational repression, but also on transcripts with a demonstrated regulation of abundance in endometriotic disease. We acknowledge that this approach may omit important molecules that are posttranscriptionally regulated by the 22 miRNAs, but that are not differentially expressed in endometriosis. Nevertheless we prefer to use this conservative approach to reduce false discovery. Predicted miRNA targets in two microarray studies (9, 10) identified pathways previously found to be critical in endometriosis (5, 6, 10). These two different mRNA data sets examined different sites of endometriosis and revealed distinct, but overlapping, transcript lists. The substantial correlation observed between these two independent comparisons lends confidence to the methodology used in evaluating the function of differentially expressed miRNAs in endometriosis.

Two distinct patient groups provided tissue for the mRNA and miRNA microarray analyses; therefore, it is possible that these individuals had distinct mRNA profiles. qRT-PCR of c-Jun confirmed that, at least for this transcript, the two independent tissue sets showed similar extents of dysregulation.

This is the first study demonstrating increased c-Jun mRNA expression by RT-PCR in ectopic vs. eutopic endometrial tissue, supporting our previous findings in a mRNA microarray study (10). Others identified reduced c-Jun mRNA levels in eutopic endometriotic compared with normal endometrial tissue (42). Endometrial c-Jun expression is induced by estrogen (43–45), a hormone required for endometriotic lesion development, whereas Danazol, an ethisterone derivative used to treat endometriosis, down-regulates c-Jun transcript levels in the lesions (46). Altogether this suggests a functional role for c-Jun at ectopic sites during endometriosis.

Functional analysis identified several molecular networks containing many transcripts linked to endometriosis. Whereas the cellular functions of these networks are consistent with the current view on endometriotic lesion pathogenesis, a comprehensive discussion of these pathways is beyond the scope of this paper. Instead, examples of the most prominent molecular networks are presented in Table 2 along with confirmed regulatory functions of the differentially expressed miRNAs, including regulation of cell migration and proliferation, wound contraction, extracellular matrix remodeling, and angiogenesis.

To our knowledge only one other study has explored miRNAs in endometriosis. Pan et al. (47) identified 48 miRNAs that were differentially expressed in eutopic and ectopic endometrium from a small study group of women with and without endometriosis; however, a direct comparison between endometriotic eutopic and eutopic tissues was not undertaken. Nevertheless, despite differences in study design, methods of analysis, measures of stringency, and RT-PCR endogenous controls, eight miRNAs concurred with the 22 miRNAs described in our analysis. This concordance underlines the likely importance of these miRNAs in the pathogenesis of endometriosis.

In summary, this study suggests that both miRNAs and the molecular pathways they target are differentially expressed between ectopic endometrial lesions and eutopic endometrium in women with endometriosis. Many of the miRNA-regulated molecular networks and biological processes identified here corroborate known endometriosis associations in the literature. These findings propose an intricate control of gene expression in endometriosis both transcriptionally and by miRNAs at the posttranscriptional level. The microarray data and molecular pathways presented here constitute a comprehensive resource on which to base future investigations into the role of specific miRNAs in endometriosis. In particular, the endometriosis-as-
associated miRNAs and the molecular pathways they target should be evaluated as new candidates for the diagnosis and treatment of endometriosis.

**Materials and Methods**

**Tissue collection and ethics approval**

All procedures for collection of tissue were approved by the Central and Northwestern Human Ethics Committee, South Australia, or the Children’s Youth and Women’s Human Research Ethics Committee, South Australia. Eutopic and ectopic endometrial tissue was obtained with written informed consent from eight women with AFS stage II–IV endometriosis during laparoscopic surgical procedures. Endometrial biopsies were collected using Pipelle suction curettes (Pipelle de Cornier, South Australia). Eutopic and ectopic endometrial tissue was obtained from eight women with AFS stage II–IV endometriosis during laparoscopic surgical procedures. Endometrial biopsies were collected using Pipelle suction curettes (Pipelle de Cornier, South Australia). Biopsies were immediately placed in RNAlater (Invitrogen, Carlsbad, CA) and stored at −80°C.

**RNA extraction**

Total RNA was extracted from endometrial tissues homogenized in Trizol solution (Invitrogen) according to the manufacturer’s instructions. RNA concentration was assessed using a Nanodrop spectrophotometer, accepting a ratio of 2.0 for sample absorbance at 260/280.

**Microarray hybridization**

The microarrays consisted of 377 anti-sense miRNA oligonucleotide probes (mirVana miRNA probe set; catalog no. 1564V1, Applied Biosystems, Foster City, CA) printed in triplicate onto epoxide-coated microscope slides (Corning, Inc., Corning, NY) with a VersArray ChipWriter Pro system (Bio-Rad Laboratories, Inc., Hercules, CA) using tungsten pins (PointTech, Barreal de Heredia, Costa Rica). Included on the array were three negative control probes, which were small-interfering RNAs of bacterial origin. For detection on the array, 5 μg total RNA was labeled by the ligation of a fluorescently modified RNA dimmer as previously described by Thomson et al.

### TABLE 2. Experimentally confirmed regulatory functions of the 22 differentially expressed miRNAs in endometriosis

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<td>miR-1 regulates apoptosis</td>
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<tr>
<td>miR-1</td>
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<td>HDAC4</td>
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<td>miR-29c</td>
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<td>TGFβ-regulated stromal proliferation</td>
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<td>ZEB1—miR-141 and miR-200b—ZEB1</td>
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<td>FN1</td>
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<td>HSDP1</td>
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<td>Cardiomyocyte apoptosis</td>
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S, Supplemental; C, fold change; Reg, miRNA regulation; ZEB1 and ZEB2, zinc finger E-box binding homeobox 1 and 2; E2F3, E2F transcription factor 3; RUNX1, runt-related transcription factor 1; FN1, fibronectin 1; CTGF, connective tissue growth factor; HSDP1, heat shock 60-kDa protein 1; EMT, epithelial to mesenchymal transition; ↑, up-regulated; ↓, down-regulated; →, positive regulation; →, inhibition.
hybridization chambers, incubated in the dark for 2 h, then washed twice in 2× standard sodium citrate (SSC)/0.025% sodium dodecyl sulfate and three times in 0.8× SSC at room temperature, and finally twice in 0.4× SSC at 4 C. Slides were scanned at 10 μm resolution with a Genepix 4000B Scanner (Molecular Devices, Sunnyvale, CA) using photomultiplier tube (PMT) settings that approximately balanced the channel intensity histograms.

Computational microarray analysis

Median spot pixel intensity values in scanned images were determined using the Spot version 3 plug-in (Commonwealth Scientific and Industrial Research Organization, Campbell Australian Capital Territory, Australia) for the statistical environment R. After subtraction of median background intensities and Loess normalization, mean intensities were log2 transformed, and ratios (Cy5/Cy3) were obtained. To select differentially expressed miRNAs, these data were analyzed using three different statistical methods. 1) A pooled correlation of triplicate spots for each miRNA was calculated for each chip using linear models and empirical Bayesian moderation of standard errors (Limma R package; Walter and Eliza Hall Institute, Parkville Victoria, Australia). 2) Alternatively, the three measurements for each transcript on each chip were analyzed using a nested ANOVA approach without empirical Bayesian moderation. 3) Data for the three measurements for each transcript on each chip (generated in method 2 above) were reanalyzed using an Ensemble learning implementation of Independent Component Analysis (ICA), which summarizes the overall transcript abundance patterns in the data. The 1% of miRNAs that contributed most strongly to the component associated with eutopic vs. ectopic tissue differences were identified. miRNAs that differentially expressed between the paired eutopic and ectopic tissues (fold change ≥ 1.5, P ≤ 0.05) according to all three methods (1–3) above were identified by Venn diagram analysis. Heatmap analysis and hierarchical clustering were performed using R.

qRT-PCR analysis of miRNA expression

Transcript levels of three up-regulated and three down-regulated miRNAs were measured by qRT-PCR in four proliferative and four secretory pair-end eutopic and ectopic endometrial total RNA samples (total n = 16).

Total RNA (5 μg) was reverse transcribed on a GeneAmp PCR System 9700 using the Taqman MicroRNA Reverse Transcription kit and primers from TaqMan MicroRNA Assays (Applied Biosystems). Water substituted for transcript and reverse transcriptase samples served as negative controls. Assay-specific cDNA and probes (TaqMan MicroRNA Assays) were combined with TaqMan 2× Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), and real-time PCR amplification was performed in triplicates using an ABI Prism 7000 (Applied Biosystems).

ΔΔCt values were calculated, and the data were normalized to hsa-miR-let-7a, which has been used as a control previously (51, 52), and to hsa-miR-let-7d. Kolmogorov-Smirnov test followed by nonparametric Mann-Whitney U test (asymptomatic significance, correlated for ties) determined the significant up- or down-regulation of ΔCt values, respectively, in ectopic compared with eutopic endometrium. Mann-Whitney U test of Ct and ΔCt [Ct(let-7a)-Ct(let-7d)] values validated the use of hsa-miR-let-7a and hsa-miR-let-7d as invariant and ubiquitous endogenous controls. The mean and SEM of fold change from eutopic endometrium was determined and the data were presented by GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA).

miRNA target prediction

Target miRNAs of the 22 differentially expressed miRNAs were determined using the mirGen web tool (53) for the algorithms TargetScan (release 3.1) (31) and PicTar (four-species conservation) (32). Targets predicted by the two-way intersection of these algorithms were further analyzed. The Ensembl gene identification nos. of putative targets retrieved from miRGen were referenced to Ensembl build 46. The convergence of these miRNA targets with miRNAs that were differentially expressed in a microarray study of ectopic and eutopic peritoneal endometrial tissue (10) was determined. According to quality control analysis in the DChip application (http://biosun1.harvard.edu/complab/dchip/), the miRNA microarray data from patient 7 contained relative outliers; therefore, this patient was excluded from further analysis. miRNA probe sets were considered to be differentially expressed at fold change ≥ ±1.5 at cyberT P ≤ 0.05. To control for potential confounding due to variation between the different patient groups contributing tissues for the miRNA and miRNA microarray analyses, a second miRNA data set comparing ovarian endometriosis with eutopic endometrium (9) was studied. miRNA targets of the 22 miRNAs that were differentially expressed in ectopic endometrium were identified in both data sets.

Permutation study of Ingenuity pathway analysis of miRNA targets

Manual pathway analysis showed that 82 molecules are associated with transcription factor MEF2C in the IPA database (http://www.ingenuity.com; May 25, 2008). Of 82 molecules, 22 are also putative targets of the 22 differentially expressed miRNAs. A permutation analysis was performed to verify the high degree of association between MEF2C and other miRNA targets in IPA: 1000 random sets of 22 miRNAs were selected from the 281 miRNAs within the Ambion human microarray probe set that were human in origin, unique, and fully annotated, and their targets were identified using the two-way intersection method described above. The intersect of the targets of each of these 1000 randomly chosen lists of 22 miRNAs, and the 82 molecules related to MEF2C in the IPA database, were calculated and plotted as histograms using R. This analysis was repeated for JUN. IPA identified 632 molecules associated with JUN (November 11, 2008), of which 147 are also putative targets of the 22 differentially expressed miRNAs.

Functional analysis of predicted mRNA targets of the 22 miRNAs

IPA was performed to identify the molecular pathways and functional groupings based on published literature, to which predicted targets of the 22 differentially expressed miRNAs belong. The targets were uploaded into IPA and overlaid on a global molecular network developed from information contained in the application. Networks of these genes were generated by IPA based on their connectivity, each ranked by a score. This score is based on the hypergeometric distribution, calculated with the right-tailed Fisher’s Exact Test, and corresponds to the negative log of this P value. Networks with a score more than or equal to 20 were selected for further analysis. Functional analysis in IPA identified the published biological functions that were most significantly associated with the genes in the network. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All lines are supported by at least one reference in literature, textbook, or from canonical information stored in the Ingenuity Pathways knowledge database.

The GOStat application (http://goastat.wehi.edu.au/cgi-bin/goStat.pl) was used to determine the biological processes annotated to the putative miRNA targets in the GO database (http://www.geneontology.org). The GOA human database was used, only considering the biological processes GO hierarchy, and GO paths with length more than or equal to 3. As a cut-off for significance, Fisher’s Exact Test (P ≤ 0.01) with Benjamini and Hochberg False Discovery Rate assessment was used to estimate the proportion of GO terms that would be identified by chance alone.

qRT-PCR analysis of c-Jun transcript levels

Standard SYBR Green qRT-PCR was carried out to detect c-Jun transcript levels in total cellular RNA from the eutopic and ectopic endometrial tissues. After treatment with ribonuclease-free deoxyribonuclease I (Ambion Turbo DNAse, Applied Biosystems; 60 min/37 C), 1 μg RNA of the individual samples was reverse transcribed using the SuperScript III Reverse Transcriptase kit (Invitrogen). Real-time PCR
was performed in a RotorGene 6000 (Corbett Life Science, Concorde, New South Wales, Australia); 95 C for 10 min, 95 C for 15 sec followed by 45 cycles at 60 C for 60 sec) on 2 μg cDNA with SYBR Green PCR Master Mix (Applied Biosystems) and 5 μg primers to c-jun [sense (5'-GGGAGGAGGCCATGAGC-3') and antisense 5'-CATGTTTG-GGCCTGAGAC-3']. These primers were specific for the published c-JUN cDNA sequence (GenBank accession no. NM_002228), designed using Primer Express version 2 software (Applied Biosystems) and optimized for use at a final concentration of 0.5 μM. The resulting 136-base amplicon spans nucleotides 1801–1943 and was analyzed by melt curve analysis to ensure lack of nonspecific products and primer dimers; 18S rRNA was analyzed in the same run with primers as recom- mended by the manufacturer (Applied Biosystems).

Linear standard curve analysis for each primer pair at a maximum 1:4096 dilution of control pooled cDNA and primer amplification efficiency (E) greater than 0.96 was obtained for all PCR experiments. c-jun data were normalized to 18S RNA and expressed in arbitrary mRNA units as median values of three independent PCR runs. The data were analyzed by two-tailed Mann-Whitney U test (95% confidence interval) in GraphPad software.

Acknowledgments

We thank Professor Julie Owens and Ms. Patricia Grant for technical advice and critical reading of this manuscript; the Adelaide Microarray Centre; Professor Yee Khong at the Department of Histopathology, Women’s and Children’s Hospital, Adelaide, Australia, for histologically dating eutopic and endometrial tissue samples relative to the menstrual cycle; staff in the Discipline of Obstetrics and Gynecology that assisted in this study; and finally all patients and nursing staff of Women’s and Children’s Hospital, Ad- elaide, Australia.

Address all correspondence and requests for reprints to: Maria Ohlsson Teague, Ph.D., and Louise Hull, MBChB, Ph.D., Research Centre for Reproductive Health, School of Paediatrics and Reproductive Health, Sixth Floor, Medical School North, University of Adelaide, South Australia 5005, Australia. E-mail: maria.teague@adelaide.edu.au (http://www.adelaide.edu.au/erch).

This work was supported by Women’s and Children’s Hospital Research Foundation Grant, Adelaide, Australia, The Arthur Wilson Fel- lowship, The Royal Australian and New Zealand College of Obstetri- cians and Gynaecologists Research Foundation, and, D.A. and J.S. Ballantyne Medical and Surgical Research Trust Grant (to M.L.H.).

Disclosure Statement: The authors have nothing to disclose.

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