Endometrial Stromal and Epithelial Cells Exhibit Unique Aberrant Molecular Defects in Patients With Endometriosis

Reproductive Sciences
2018, Vol. 25(1) 140-159
© The Author(s) 2017
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719117704905
journals.sagepub.com/home/rsx

Philip C. Logan, PhD¹ , Pamela Yango, BS¹, and Nam D. Tran, MD, PhD¹

Abstract

Context: Endometriosis is a chronic inflammatory disease that causes pain and infertility in women of reproductive age. **Objective:** To investigate the pathologic pathways in endometrial stromal and epithelial cells that contribute to the manifestation of endometriosis. **Design:** In vitro cellular and molecular analyses of isolated eutopic endometrial stromal and epithelial cells. **Methods:** Eutopic stromal and epithelial cells from endometriotic and normal patients were isolated by fluorescence-activated cell sorting for paired sibling RNA sequencing and microRNA microarray. Aberrant pathways were identified using ingenuity pathway analysis networks and confirmed with in vitro modulation of the affected pathways in stromal and epithelial cell cultures. **Results:** Both stromal versus epithelial cell types and paired endometriotic versus normal samples exhibited distinct hierarchical clustering. Compared to normal samples, there were 151 and 215 differentially expressed genes in the endometriotic stromal and epithelial populations, respectively, and concomitantly 9 and 16 differentially expressed microRNAs. Overall, endometriotic stromal and epithelial cells revealed distinct defects. In endometriotic stromal cells, key decidualization genes *Zinc finger E-box Binding protein 1 (ZEB1)*, *Heart And Neural crest Derivatives expressed 2 (HAND2)*, *WNT4*, and *Interleukin 15 (IL-15)* were found to be downregulated and *Periostin (POSTN)* and *Matrix Metalloproteinase 7 (MMP7)* were upregulated. Specifically, *ZEB1* was downregulated in stromal cells by aberrant elevation in miR-200b. In contrast, *ZEB1* was found to be upregulated in endometriotic epithelial cells through associated upregulation of transforming growth factor β 1 (TGF β 1), inducer of the TGF β 1–Bone Morphogenetic Protein 2 (BMP2)–MMP2–Prostaglandin-endoperoxide Synthase 2 (COX2)–*ZEB1* pathway, which activates epithelial–mesenchymal transition. **Conclusion:** Manifestation of endometriosis involves dysregulation of unique molecular pathways within the diseased endometrial stromal and epithelial cells in the endometrium. Targeting the cell type–specific defects may offer a novel approach to treating endometriosis.

Keywords

decidualization, epithelial–mesenchymal transition, microRNA, TGF β 1, *ZEB1*

Introduction

The extrauterine invasion and growth of estrogen-dependent endometrial cells is the clinical hallmark of patients with endometriosis.¹⁻³ It is an inflammatory disease that affects approximately 10% of women of reproductive-age,^{4,5} with symptoms such as chronic pelvic pain, dysmenorrhea, and infertility, which often precede the diagnosis of endometriosis by an average of 11 years.^{3,6,7} Even when diagnosed, there is no current effective and definitive treatment beyond temporizing surgical or medical intervention.

Retrograde menstruation of eutopic endometrial tissues, stem cell origin, coelomic metaplasia, or developmental origins during fetal development have all been hypothesized to be potential causes of endometriosis; however, the precise pathophysiology of endometriosis remains to be understood.⁸⁻¹⁶ Recently, molecular profiling studies examining the disease from a variety of approaches have offered critical insights into the basic understanding of endometriosis.¹⁷⁻²² Microarray

studies have revealed differential gene expression in the eutopic endometrium between endometriotic and normal, between ectopic and eutopic tissues, and comparisons made between the phases of the menstrual cycle.^{17,23-27} Similarly, epigenetic mechanisms, which often mediate environmental factors,²⁸⁻³¹ such as DNA methylation,³²⁻³⁷ histone modification,³⁸⁻⁴¹ long noncoding RNA,^{22,42} and microRNA,⁴³⁻⁴⁹ have also been investigated.

However, there are inconsistencies and a general lack of consensus among studies for specific genes and microRNAs

¹ Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, San Francisco, CA, USA

Corresponding Author:

Nam D. Tran, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, 513 Parnassus Ave, San Francisco, CA 94143, USA.
Email: namdtran@gmail.com

involved in the reported aberrant molecular pathways.^{17,45} Perhaps this lack of clarity is due to the complex nature of this disease and the diversity of the endometrial tissues used in these studies.¹⁷ Specifically, molecular studies often use tissues collected from different phases of the menstrual cycle and predominantly utilize whole endometrial biopsies consisting of mixed populations of endothelial, stromal, and epithelial cells for analyses.^{25,46,50-52} While informative, it is challenging to resolve the specific impact of the menstrual cycle and how individual cell types within the endometrium contribute to the overall pathophysiology of the disease.⁵³⁻⁵⁵ Since endometrium from patients was shown to share similar defects to ectopic endometrium (although there are some differences), the studies of isolated stromal and epithelial cells from the eutopic endometrium could minimize the heterogeneity seen among current studies.^{23,51}

Endometrial stromal and epithelial cells have separate yet important interacting functions. Stromal cells are fibroblast-like cells that differentiate into larger, rounder decidual cells during the secretory phase of the menstrual cycle in order to be receptive and nourish an embryo ready to implant inside the endometrium.^{56,57} Failure of stromal cells to decidualize properly has consequences such as impaired fertility, placenta accreta, poorly developed placenta, or miscarriage.⁵⁸⁻⁶⁰ Meanwhile, epithelial cells form a protective lining of the lumen interface of the endometrium and glandular ducts and are the point of attachment for an embryo to implant.⁶¹ A 2-way paracrine interaction exists between stromal and epithelial cells, in particular, retinoic acid signaling from stromal to epithelial cells, and conversely, the Hedgehog pathway paracrine signaling from epithelial cells to stromal cells that regulates *WNT family member 4 (WNT4)*, a critical protein for decidualization.⁶²⁻⁶⁶

In this study, paired eutopic endometrial stromal and epithelial cells from diseased (endometriotic) and normal endometrial biopsies were used to investigate the cell type-specific aberrant molecular pathways involved in the pathogenesis of endometriosis.

Materials and Methods

Tissue Collection

Endometrial tissue was collected, after informed written consent, from normal patients and patients with endometriosis (Supplementary Table 1) under a protocol approved in writing by the Committee on Human Research of the University of California, San Francisco (UCSF). All patients had regular menstrual cycles and were in the proliferative phase. Endometriotic tissue was confirmed by laparoscopy, with histologic evaluation of normal and stage I to IV endometriosis. Staging of endometriosis was defined according to the revised American Fertility Society classification system. Exclusion criteria were tissues from patients with fibroids, endometrial polyps, pregnancy, or any hormonal medications known to interfere with the menstrual cycle either directly on the uterus or centrally for at least 3 months before tissue collection.

Tissue Digestion

Endometrial tissue from more than 29 patients was digested according to established protocol. The fresh tissue was minced and digested with 3.15 mg/mL collagenase I (Worthington Biochemical, Lakewood, New Jersey) and 40 µg/mL deoxyribonuclease I (DNase I; Sigma Aldrich, St Louis, Missouri) in digestion media (Dulbecco's minimum essential medium [DMEM]/F12 phenol red-free supplemented with 10% fetal bovine serum [FBS]; Gibco, Life Technologies, Carlsbad, California) and 1% antimycotic (penicillin, streptomycin, fungizone [amphotericin B]; Cell Culture Facility, UCSF) on a rotator at 37°C for 1 hour. The digestion was filtered through a 40-µm cell strainer. Both the flow-through fraction and the backwash fraction were collected in 10 mL digestion media and centrifuged. The first fraction pellet was reconstituted and the second fraction incubated with 0.8 mg/mL collagenase II (Worthington) and 40 µg/mL DNase I at 37°C for 20 minutes and centrifuged, after which the pellet was incubated with 2 mL Accutase (Life Technologies) at 37°C for 10 minutes. This fraction was filtered through a 70-µm cell strainer, centrifuged, and the pellet reconstituted.

Fluorescence-Activated Cell Sorting of Human Endometrial Stromal and Epithelial Cells

To sort into stromal fibroblast and epithelial cells, digested cells were labeled with antibody staining, incubated for 30 minutes at 37°C, and sorted by fluorescence-activated cell sorting (FACS) AriaII UV, with FACSDiva software (BD Biosciences, San Jose, California; DRC Center Grant [NIH P30 DK063720] Parnassus Flow Cytometry Core, UCSF). Otherwise, all cells were kept on ice during processing and after the FACS sorting. Antibody staining to isolate stromal cells was CD90 (BioLegend, San Diego, California) and epithelial cells was EpCAM PerCP-Cy5.5 (CD326, cat #347199; Becton Dickinson, Franklin Lakes, New Jersey). All cells were labeled with FITC CD146 (cat # 560846; BD Pharmingen, San Jose, California) to remove endothelial cells, Pacific Blue CD45 (cat # 304029; BioLegend) to remove leukocytes, and Sytox Blue (Life Technologies) to remove dead cells. The FACS cell sort was analyzed using FlowJo v9.6 (FlowJo, Ashland, Oregon). (Fluorescence-activated cell sorting selects large quantities of cells purely compared to laser capture of cells).

Isolation of Stromal and Epithelial Cells

To compare diseased versus normal endometrial stromal and epithelial cells, these 2 cell types were isolated from endometrial biopsies from both diseased and normal patients (Figure 1A). Only live nonleukocytic endometrial cells were gated for initial analysis. Subsequently, only enriched stromal fibroblast cells (CD90⁺) and epithelial cells (EPCAM⁺) were collected after the exclusion of endothelial cells (Figure 1B). Immunocytochemistry confirmed the purity of stromal and epithelial cells with vimentin staining of stromal cells (C9080; Sigma Aldrich) and pan-cytokeratin of epithelial cells (M0821; DakoCytomation, Santa

Figure 1. A, Isolation of stromal and epithelial cells from endometriotic and normal tissue for sibling-paired RNA-Seq and micro-RNA microarray analyses; each sample was independently sorted and analyzed. B, Isolation of stromal fibroblasts (CD90⁺) and epithelial (EpCAM⁺) cells by fluorescence-activated cell sorting (FACS) and analyzed by FlowJo v9.6. Only single, CD45⁻/CD146⁻ live cells were gated for stromal fibroblast and epithelial cell isolation (leukocytes and endothelial cells were excluded). FSC-A indicates forward scatter area; SSC-A, side scatter area.

Clara, California). RNA was collected from the sibling stromal and epithelial population from each patient sample, and each RNA sample was further divided equally for paired RNA and microRNA analyses with RNA sequencing (RNA-Seq) and microRNA microarray, respectively (Figure 1A).

Cell Culture

Stromal cells were cultured in DMEM/F12 phenol red-free 10% FBS media (Gibco, Life Technologies) with 1% antimycotic (penicillin/streptomycin/fungizone). Epithelial cells were cultured in defined keratinocyte serum-free media supplemented with L-glutamine and initially 28% FBS (Life Technologies). All cells were incubated at humidified 37°C and 5% CO₂. Cell cultures of both stromal (n = 4) and epithelial cells (n = 6) were used for transient transfection studies of microRNA and their target genes.

RNA Isolation

Total RNA was isolated for RNA-Seq and microRNA microarray by RNeasy Plus Mini Kit (Qiagen, Valencia, California). High-quality RNA for both assays was confirmed on a Bioanalyzer 2100 (Bioanalyzer; Agilent Technologies, Santa Clara, California), and the samples had a minimum RNA integrity number of 8.8. Otherwise, total RNA for quantitative polymerase chain reaction (qPCR) was isolated by TRI-reagent (Sigma-Aldrich) following the manufacturer's instructions.

RNA Sequencing Expression Profiling

Endometrial cells from 3 patients with endometriosis and 3 normal participants were sorted by FACS into stromal and epithelial cells (Figure 1A). Cell type-specific diseased and normal samples were processed individually during RNA isolation. Each sample was divided equally for RNA-Seq and microRNA microarray. TopHat and Cufflinks were used to process genome-wide sequencing data and Cuffdiff to obtain values for FPKM (fragments per kilobase of exon per million fragments mapped). Significant messenger RNA (mRNA) differential expression was defined as >2-fold change and Cuffdiff $P < .05$.

Pathway Analysis of Different Gene Networks in Endometriotic Stromal and Epithelial Cells

Pathway analysis of the differentially expressed genes profiled by RNA-Seq from stromal and epithelial cells was performed using ingenuity pathway analysis (IPA; Qiagen, Redwood City, California, www.qiagen.com/ingenuity). The DAVID Bioinformatics Resource (v6.7) was also used to analyze the stromal and epithelial data sets (Database for Annotation, Visualization and Integrated Discovery. [NIAID] NIH. Huang et al 2009).

microRNA Microarray Expression Profiling

The microarray was conducted on an Affymetrix GeneChip miRNA 3.0 Array (Affymetrix, Santa Clara, California). Unique reads were aligned to human microRNA sequences from miRBaseGv17 (www.mirbase.org). The microarray detected more than 1300 microRNAs (Supplementary Table 2). Significant microRNA differential expression was defined as ≥ 1.5 -fold change and Students t test $P \leq .05$.

Figure 2. A, Dendrogram of hierarchical clustering analysis (HCA) of messenger RNA (mRNA) data from RNA-Seq; q1-q4 are sample queries. B, Venn diagram of mRNA expression from RNA-Seq of endometriotic compared to normal stromal and epithelial cells; selection by >2-fold change, CuffDiff, $P < .05$. C, Endometriotic stromal genes from RNA-Seq data set validated by quantitative polymerase chain reaction (qPCR), normalized with 18S RNA, $n = 4$. D, Endometriotic epithelial genes from RNA-Seq data set validated by PCR, normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), $n = 5$. Mean (Standard deviation). * $P \leq .05$, ** $P \leq .01$.

Real-Time qPCR for mRNA and microRNA

Validation of RNA-Seq and microarray was performed by qPCR of stromal cell differential mRNA ($n = 4$), microRNA ($n = 3$), and epithelial mRNA ($n = 5$), and microRNA ($n = 6$). Total RNA was converted to complementary DNA (cDNA) by qScript SuperMix (Quanta Biosciences, Gaithersburg, Maryland) for mRNA expression and qScript microRNA cDNA Synthesis (Quanta Biosciences) for microRNA by following the manufacturer's instructions. FastStart SYBR-Green ROX (Roche Diagnostics, Indianapolis, Indiana) was used for mRNA expression and PerfeCTa SYBR Green Supermix Low-ROX (Quanta Biosciences) used for microRNA expression. Quantitative PCR was performed in an Applied Biosystems ViiA7 real-time PCR system (Life Technologies). Primers used for qPCR are listed in Supplementary Table 3. The $\Delta\Delta CT$ method was used to calculate the relative quantity of transcripts. The reference genes for mRNA qPCR were selected as suitable for 2 different cell types from our previous studies: *RNA18S5* for stromal, *Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH)* for epithelial cells, and *Small Nucleolar RNA C/D box 44 (SNORD44)* for microRNA qPCR.

microRNA Target Genes

The selection of predicted mRNA target genes of miR-200b and miR-204 for stromal cells and miR-504 and miR-1827 for epithelial cells from our microarray data was based on DIANA-lab MicroT-CDS (www.diana.imis.athena-innovation.gr/DianaTools/index.php) and TargetScanHuman v6.2 (www.targetscan.org). The predicted targets of microRNAs were matched with our RNA-Seq data set of differentially expressed mRNA (Supplementary Table 4). Transfection conditions for mimics (overexpression) and antagomirs (inhibition) of selected microRNAs (Supplementary Table 5) were 15 nM for mimics, incubated for 48 hours, and 50 nM for antagomirs, incubated for 72 hours. The transfection reagent DharmaFECT 1 (T-2001-03; GE Dharmacon, Lafayette, Colorado) was applied at 1 μ L per transfection in 6-well plates. Nontargeting mimic and hairpin inhibitor were negative controls.

Statistical Analysis

Data were analyzed and graphed using GraphPad Prism v6.0.5 for Windows (GraphPad Software, La Jolla, California, www.graphpad.com). Results are expressed as mean (standard

deviation). Statistical significance was determined by Student unpaired, 2-tailed *t* test and for groups of 3 or more by 1-way analysis of variance with Benjamini-Hochberg multiple testing correction for false discovery rate: for RNA-Seq and microarray assays; $n \geq 3$ samples for each experiment.

Results

Differential Expression of Genes in Endometriotic Stromal and Epithelial Cells

To determine the differential gene expression between endometriotic and normal stromal cells, and similarly for epithelial cells, RNA-Seq was performed on paired sibling samples of stromal and epithelial cells. On average, there were 51 501 530 and 45 364 780 total reads of >23 000 genes from stromal and epithelial samples, respectively. Hierarchical clustering analyses revealed that there were distinct, differentially expressed genes between stromal and epithelial cells, regardless of whether they were endometriotic or normal (Figure 2A). Additionally, distinct clustering was also observed between endometriotic and normal stromal and epithelial endometrial cells (Figure 2A). Endometriotic stromal and epithelial cells had 151 (52 upregulated) and 215 (80 upregulated) differentially expressed genes, respectively (Figure 2B; Table 1). There were only 5 common differentially regulated genes between diseased stromal and epithelial cells (upregulated: *Colony Stimulating Factor 3 (CSF3)*, *Serpin family A member 1 (SERPINA1)*, *ASH1 Like histone lysine methyltransferase (ASH1L)*⁶⁷; downregulated: *Phosphoinositide-3-Kinase Regulatory subunit 1 (PIK3R1)*, *latent TGFβ1-binding protein 4 (LTBP4)*; Figure 2B). *Zinc finger E-Box Binding homeobox 1 (ZEB1)*, *Heart And Neural crest Derivatives expressed 2 (HAND2)*, and *C-X-C motif Chemokine Ligand 12 (CXCL12)* were confirmed to be downregulated, while *Periostin (POSTN)*, *SERPINA1*, and *CSF3* were upregulated in diseased stromal cells by qPCR, consistent with RNA-Seq data (Figure 2C). The qPCR also confirmed upregulation of *ZEB1*, *Integrin subunit beta 6 (ITGB6)*, *CSF3*, *Bone Morphogenetic Protein 2 (BMP2)*, and *Fms related tyrosine kinase 1 (FLT1)* and downregulation of *PIK3R1* in diseased epithelial cells (Figure 2D). Interestingly, *ZEB1* was downregulated in diseased stromal cells, while it was upregulated in diseased epithelial cells.

Differential Expression of microRNAs in Endometriotic Stromal and Epithelial Cells

To determine the specific microRNA expression between diseased and normal stromal and epithelial cells, the same RNA samples that were used for RNA-Seq were also used for microRNA microarray for paired microRNA-mRNA analyses. Hierarchical clustering of these data revealed that differentially expressed microRNAs in stromal cells were distinct from microRNAs in epithelial cells and that differentially expressed microRNAs in endometriotic samples were distinct

Table 1. Differentially Expressed Genes in Endometriosis From RNA-Seq: 151 Endometriotic Stromal Genes and 215 Epithelial Genes.^a

Stromal Genes	Log2 Change
ABCA8	-2.593
ABCC8	-3.511
ADAMTS15	-2.024
ADAMTS5	-1.818
ADCY1	-1.983
AHNAK2	-1.364
ALDH1A3	-1.548
ALDH1L2	-1.405
ALPK3	2.736
ANKRD13B	-2.648
AP2A1	-1.517
APBB1IP	-3.899
ASH1L	2.699
ATP8A2	-2.044
BCAT1	-1.321
BMPER	-2.231
BRINP1	-3.483
BTBD2	-1.770
BTBD3	-1.359
C10orf128	4.444
C1orf95	-3.335
C2	-2.545
C20orf27	-2.313
C3	-1.644
CBS	-2.425
CD93	1.683
CDH6	1.873
CFD	-4.148
CILP	-3.583
CLDN11	2.847
CLEC3B	-3.926
COL17A1	-6.048
COL24A1	1.537
COL4A5	-1.956
COL4A6	-4.199
COLEC11	-2.198
CPE	2.253
CRABP2	1.616
CREB5	1.143
CSF3	2.672
CXCL12	-1.642
CYP11A1	4.355
DEPTOR	-3.093
DIO3	-3.555
DLL4	1.922
DUSP10	1.735
EMILIN2	-1.241
EMR2	1.452
EPHA7	1.962
ETV5	1.466
FAM134B	-1.702
FBLN2	-1.631
FERMT1	-2.387
FGF10	-3.295
FMNL3	1.528
FNDC1	2.163
FOXF1	1.999

(continued)

Table 1. (continued)

Stromal Genes	Log2 Change
G0S2	1.782
GABRA2	-2.857
GALNT13	-2.554
GFRA2	3.334
GLB1L2	-4.410
GNG4	-1.477
GPBAR1	-2.955
GPR116	2.633
GPR133	-2.057
GPR64	-2.186
GRIA4	-2.102
H6PD	-1.248
HAND2	-1.388
HSDL2	-1.681
HSPB6	-2.728
IGBP1P1	4.444
IGFBP2	-1.445
IL15	-2.158
ITGB8	-1.196
KALI	-2.639
KCND3	-3.827
KCNH1	-2.149
KCNK3	-2.210
LAPTM5	2.705
LGI2	-2.298
LIMCH1	-1.817
LRRC15	-2.763
LTBP4	-1.659
LYNX1	-3.025
MAOB	-1.831
MICAL1	-1.303
MMP7	2.081
MPZL2	3.159
MROH6	-2.577
MTR	-1.293
MYD88	-1.295
MYLK3	-1.826
MYOCD	-1.867
NMB	1.925
NPW	-3.533
NRARP	2.329
NRG1	1.751
NRXN1	-2.261
OAS2	-1.956
PAPLN	-1.291
PCDH20	-3.091
PDE3A	1.825
PDGFA	1.847
PDLIM3	2.191
PFKFB4	2.181
PI15	3.574
PIK3R1	-1.145
PILRA	-3.592
PIM2	2.249
PLA2G7	-2.310
PLCBI	3.325
PLCD1	-2.120
PLCLI	-1.446
PLXND1	-1.507

(continued)

Table 1. (continued)

Stromal Genes	Log2 Change
POSTN	1.973
PPMIH	-1.617
PPP1R14A	-2.849
PPP4R4	3.947
PROK1	-5.321
PTCHI	-1.619
PTGDS	-1.830
PTPRB	1.853
PYGL	-1.741
RAPGEF4	2.370
RASD1	-1.462
RASGRP3	2.266
REXO1	3.576
RPRM	-3.173
S100A7	4.444
SCARA5	-6.773
SDK1	-1.382
SELE	1.910
SERPINA1	3.799
SHB	1.492
SLC2A8	-2.747
SLC40A1	-1.515
SMIM3	1.509
SPATA18	-2.621
STEAP1	2.559
SULF2	-1.292
TMEM132B	-1.692
TMEM196	-3.447
TMEM37	-2.290
UBA7	-5.969
UCN2	1.774
WNT4	-2.081
XIRP1	2.145
ZC3H12C	1.229
ZNF703	-1.630
Epithelial genes	Log2 Change
ABCA6	-2.541
ABCD1	-2.951
ABL2	1.543
ACPP	-2.450
ACTG2	-3.924
ADAMTS1	-1.221
ADAMTS8	-6.935
AKR1C2	3.146
ALDH3B2	-4.806
ALOX5	1.839
ALPL	-1.593
ANO6	1.160
AOX1	-2.827
AQP9	2.792
ARHGAP15	-4.444
ARNTL2	1.206
ASH1L	2.792
ASS1	1.359
ATP10B	-4.313
ATP6V0E2	-2.908
AXL	2.646
BMP2	3.687
C14orf183	4.444

(continued)

Table 1. (continued)

Stromal Genes	Log2 Change
Clorf64	-4.444
C2orf54	2.703
CABYR	-2.156
CAMP	-4.444
CAPN14	2.627
CAPN6	-2.525
CBX4	-1.776
CCBL1	-3.111
CCDC144B	1.692
CD36	-4.850
CD40	3.180
CDK6	2.611
CENPF	-2.349
CES4A	-2.668
CHST10	-2.045
CIC	-1.832
CLGN	-1.896
CMYA5	-2.098
COL1A2	-3.050
COL27A1	2.500
COL6A2	3.924
COL6A3	2.396
CREB3L1	-2.739
CSF3	2.628
CST6	2.768
CXCL5	2.128
CXorf27	4.444
CYP26A1	-4.647
DFNB31	-1.719
DLX5	-1.397
DNAJA4	-1.319
DPP4	-1.609
DPYSL3	2.766
DUOX1	-2.608
DUOXA1	-2.589
DYNC111	-2.271
ECI2	-2.016
EDNRB	-2.450
EEF2K	-1.403
ELK3	1.259
ELMSAN1	-1.384
ELP3	-2.000
EML5	-2.105
EMPI	1.247
EMP3	2.144
EPN3	-3.353
ETV1	-2.130
FAM129A	1.802
FAM171B	-2.418
FAM172A	3.709
FGB	2.721
FGF2	2.273
FILIP1	-3.850
FJX1	2.023
FLT1	2.257
FMOD	2.986
FNI	2.144
GCNT1	1.529
GJB4	3.413

(continued)

Table 1. (continued)

Stromal Genes	Log2 Change
GLYATL2	-5.294
GLYATL3	-4.444
GMPR	-2.515
GPR141	4.444
GRAMD1C	-1.629
GREM2	-2.130
HEY1	-2.369
HGD	-2.045
HPGD	-1.925
HS6ST1	-1.659
HSD11B2	-2.567
HSD17B2	-2.184
HSPA6	-1.878
HYAL1	-2.535
IDH1	-2.126
IGF2	2.123
IGFBP5	2.024
IL1B	2.942
IL1R2	2.076
IL1RN	1.675
IL20RA	-2.481
ITGB6	1.352
KAT2B	-1.876
KCTD12	2.115
KLF2	-2.404
KRT13	2.134
KRT17	1.444
KRT7	1.294
LSAMP	2.916
LTBP4	-2.107
MAOA	-1.668
MAP2K6	-2.859
MARCKS	1.322
MESPI	-4.444
MMP2	2.872
MMP26	-1.956
MSX1	-1.273
MT1G	-2.416
MT1H	-3.334
MT1M	-2.949
MTSS1	-2.023
MUC5B	3.391
MXRA5	1.560
NAV3	2.895
NDC80	-3.144
NEK6	-1.323
NLRP5	-4.444
NME4	-1.641
NOS2	4.861
NPR3	-4.013
NQO1	-1.331
NRP2	2.272
OGDHL	-2.263
OPRK1	-2.528
PAPSS1	-1.229
PAX2	-1.976
PCSK5	1.925
PEBP4	-4.444

(continued)

Table 1. (continued)

Stromal Genes	Log2 Change
PER1	-1.715
PIK3R1	-1.931
PIK3R2	-1.558
PKHD1L1	-2.633
PLLP	-1.501
PLXNA4	-4.199
PRICKLE1	-2.119
PROL1	-4.444
PSG9	-5.337
PTGS2	1.792
RAB20	-2.004
RAB31	1.925
RAP1GAP	-2.171
RBPI	1.920
RNASE1	-3.146
RYS3	-2.010
SAPCD2	-2.872
SCGB1D2	-2.090
SCGB1D4	-4.296
SCNN1B	2.356
SERPINA1	1.201
SERPINA5	-3.252
SERPINB2	5.797
SERPINB5	3.364
SERPINE2	2.326
SFTPA2	-4.444
SHISA2	2.114
SHISA8	-5.245
SLC15A2	-2.851
SLC25A1	-1.564
SLC25A15	-3.775
SLC25A38	-1.395
SLC25A48	-4.444
SLC26A9	2.495
SLC39A8	-2.066
SLC43A1	-3.262
SLC46A2	-4.623
SLC47A1	2.041
SLC6A14	2.618
SLC7A4	-3.340
SLC7A8	-1.826
SLCO2A1	2.901
SMAD9	-1.745
SPARC	2.106
SPDEF	-2.606
SPOCD1	2.612
SPOCK1	1.774
SPON1	-1.533
SQLE	-1.358
ST3GAL3	-2.045
ST6GALNAC1	-2.143
STEAP4	-1.486
STRA6	3.899
SUFU	-2.606
TCEA3	-3.842
TEX36	4.444
TGFBI	4.850
TGM2	1.806
THEM4	-1.444

(continued)

Table 1. (continued)

Stromal Genes	Log2 Change
TMEM101	-1.617
TMEM132C	-2.257
TMEM252	-3.294
TMEM42	2.413
TOP2A	-2.279
TPD52L1	-1.420
TRPC6	-2.375
TRPM8	-2.202
TSPAN12	-1.461
TTLI1	-3.297
UBAP2	3.475
USP54	-1.223
WFS1	-1.482
ZEB1	2.103
ZNF319	-2.254
ZNF589	-1.718

^aSelection by >2-fold change, $P < .05$.

from microRNAs in normal samples (Figure 3A). Compared to normal samples, there were 9 (6 upregulated) and 16 (8 upregulated) differentially expressed microRNAs in diseased stromal cells and epithelial cells, respectively (Figure 3B; Table 2). Specifically, miR-200b and miR-204 were confirmed to be upregulated in diseased stromal cells, while miR-1827 was confirmed to be upregulated and miR-504 downregulated in diseased epithelial cells by qPCR (Figure 3C).

Aberrant Pathways Involved in Endometriosis

Potential molecular pathways involved in both endometriotic stromal and epithelial cells were evaluated by IPA software and by DAVID Bioinformatics (Table 3). Based on the differentially expressed genes between the endometriotic and the normal stromal populations, IPA identified 44 genes involved in the *Protein Kinase B (Akt)/Extracellular signal-Regulated Kinases (ERK)/Mitogen Activated Protein Kinase 14 (P38MAPK)/Tumor Necrosis Factor (TNF)/Nuclear Factor kappa B (NF-κB)* decidualization pathways, such as *WNT4*, *HAND2*, *POSTN*, *Interleukin 15 (IL-15)*, *Complement Factor D (CFD)*, *SERPINA1*, and *Matrix Metalloproteinase 7 (MMP7)* (Figure 3D). The IPA identified the differentially expressed genes within the diseased stromal population that function to interfere with normal immune cell trafficking ($z = -2.190$), with *myeloid differentiation primary response 88 (MYD88)* as the master upstream regulator together with *IL-15*, *Complement C3 (C3)*, *Plexin D1 (PLXND1)*, and *CXCL12*. Additionally, the differentially expressed *MMP7*, *POSTN*, *SERPINA1*, and *Insulin like Growth Factor Binding Protein 2 (IGFBP2)* genes also function to modulate cell migration (2.186; Table 3). DAVID classified the main altered functional groups of stromal cells into signal (64 genes), extracellular (29 genes), cell adhesion (17 genes), vasculature development (12 genes), and

Figure 3. A, Dendrogram of hierarchical clustering analysis of microRNA data from microRNA microarray, q1 to q4 are sample queries. B, Venn diagram of microRNA expression from microRNA microarray of endometriotic stromal and epithelial cells; selection by >1.5-fold change, $P \leq .05$. C, MicroRNAs from microRNA microarray of endometriotic stromal ($n = 3$) and epithelial cells ($n = 6$) validated by quantitative polymerase chain reaction (qPCR), normalized with SNORD44. D, Ingenuity pathway analysis (IPA) of Akt/ERK/P38MAPK/TNF/NF- κ B network of endometriotic stromal messenger RNA (mRNA). E, Venn diagram of mRNA expression from RNA-Seq of endometriotic stromal and epithelial genes with subsets of predicted microRNA target genes. F, The IPA analysis of microRNA predicted target gene network in endometriotic epithelial cells. G, The IPA analysis of mRNA transforming growth factor β 1 (TGF β 1)/interleukin 1B (IL-1B) network in endometriotic epithelial cells. H, The IPA analysis of microRNA predicted target gene network in endometriotic epithelial cells. Shaded genes are statistically significant. Shading intensity of a gene corresponds with the degree of up- or downregulation in endometriosis. Red-colored genes are upregulated in endometriosis and green-colored genes are downregulated. Mean (standard deviation). * $P \leq .05$.

Figure 3. (continued)

Table 2. microRNA Differential Expression in Endometriotic Stromal and Epithelial Cells.^a

Stromal		Epithelial	
Up	Fold Change	Up	Fold Change
miR-200b	2.93	miR-936	1.57
miR-204	2.19	miR-1827	1.98
miR-629	1.77	miR-3141	3.76
miR-3926	1.49	miR-3938	1.51
miR-3928	1.69	miR-4266	1.59
miR-4670	1.66	miR-4450	1.83
		miR-4522	1.64
		miR-4792	2.27
Down		Down	
miR-548al	0.28	miR-375	0.21
miR-1297	0.52	miR-504	0.53
miR-4795	0.57	miR-548n	0.54
		miR-601	0.61
		miR-603	0.56
		miR-3136	0.51
		miR-3176	0.59
		miR-4476	0.49

^aSelection by >1.5-fold change, $P \leq .05$.

neuropeptide signal (7 genes) (Table 3; Supplementary Table 6). Diseased stromal cells have 5 differentially expressed microRNAs that have 33 predicted target mRNAs within the same differentially regulated genes found in RNA-Seq (Supplementary Table 4), represented as a subset in the Venn diagram (Figure 3E). The predicted TNF/ERK/p38MAPK/NF- κ B pathways based on the differentially expressed microRNAs and their target mRNAs in endometriotic stromal cells (Figure 3F) suggests that upstream microRNA regulation plays an important role in impaired decidualization, with key genes *Myosin Light chain Kinase 3 (MYLK3)*, *ITGB8*, *Patched 1 (PTCH1)*, and *WNT4* being downregulated. *WNT4* regulates the critical *Forkhead box O1 (FOXO1)* transcription factor via the canonical β -catenin signaling pathway.⁶⁶

Of the 215 differentially regulated genes from the diseased epithelial cells, there were 87 genes identified from the transforming growth factor β 1 (TGF β 1) family, involved in the TGF β 1/interleukin 1B (IL-1B) pro-inflammatory pathways (Figure 3G). Additionally, IPA identified an overall increase associated with cancer development ($z = 2.268$), with TGF β 1 as an upstream regulator, which encompassed tumor cell proliferation (2.537), leukocyte migration (2.608), cell migration (2.803), and vascularization (2.420; Table 3). The critical genes *TGF β 1*, *ZEB1*, *BMP2*, *MMP2*, and *Prostaglandin-endoperoxide Synthase 2 (PTGS2, COX2)*, which were all upregulated in RNA-Seq, activate the epithelial–mesenchymal transition (EMT) pathway (Table 1). DAVID classified the 215 differentially regulated genes into the main functional gene groups of signal, (48 genes) extracellular, (35 genes) and vasculature development, (11 genes) which is similar to stromal cells but with a different set of genes (Table 3; Supplementary Table 6). The differentially expressed microRNAs in the diseased epithelial cells shared 97 predicted target genes found to be differentially expressed based on RNA-Seq analyses of the diseased epithelial cells (Supplementary Table 4). Of these 97 target genes, 24 target genes represent the main functional groups from the IPA analysis of the TGF β 1/IL-1B/Akt/ERK pathway (Figure 3H).

Target Genes of miR-200b and miR-204 in Stromal Cells

To investigate and confirm the target genes of differentially expressed microRNAs involved in the aberrant pathways described, the levels of miR-200b and miR-204 within the diseased and normal stromal cells were modulated with specific mimics and antagomirs by transient transfection. The qPCR confirmed that mimics and antagomirs could modulate the levels of miR-200b and miR-204 within the stromal cells in vitro as expected (Figure 4A). Upregulation of miR-200b by mimic downregulated the expression of target genes *ZEB1*, *PTCH1*, *Forkhead box F1 (FOXF1)*, and *CAMP Responsive Element Binding protein 5 (CREB5)*; however, the sequestration of miR-200b through antagomir upregulated only *ZEB1*

Table 3. Network and Gene Analysis of Differentially Expressed mRNA, from RNA-Seq of Endometriotic Stromal and Epithelial Samples, by Ingenuity Pathway Analysis (IPA) and DAVID.

Ingenuity Pathway Analysis				
z Score	Function	Genes		
Stromal				
−2.190	Immune cell trafficking decrease	MYD88 upstream		
−2.366	Antigen-presenting cell movement decrease	C3, CXCL12, IL15, MYD88, PLXND1, PTGDS, SELE		
−2.000	Mast cell migration decrease	C3, CXCL12, IL15, PIK3R1		
−2.216	Macrophage cell movement decrease	C3, CXCL12, IL15, PLXND1, PTGDS		
−2.177	Phagocyte maturation decrease	CSF3, IL-15, MYD88, PIK3R1, SERPINA1		
−2.219	Inflammatory disease decrease	C3, CXCL12, DLL4, MYD88, PIK3R1		
2.321	Cell morphology increases cell size	CBS, DEPTOR, PIM2, PLCB1, UCN2, IGFBP2		
2.186	Muscle cell migration increase	IGFBP2, MMP7, MYOCD, POSTN, SERPINA1		
Epithelial				
2.268	Cancer increase	TGFβ1 upstream		
2.537	Tumor cell proliferation increase	AXL, CST6, IL1RN, MMP2, NOS2, PTGS2 (COX2), ZEB1		
2.420	Vascularization increase	ADAMTS1, ALOX5, FGF2, IGF2, IL1B, NOS2, NRP2, SPARC		
2.803	Cell migration increase	ALOX5, AXL, CDK6, CHST10, CXCL5, FGF2, FMOD, GCNT1, HEY1, IGF2, IL1B, NOS2, NQO1, NRP2, RAPIGAP, SPARC, SPDEF, TGM2		
2.608	Leukocyte migration increase	ALOX5, AQP9, CXCL5, FGF2, GCNT1, IGF2, IL1B, NOS2, NQO1, NRP2, RAPIGAP, SPARC, SPDEF, TGM2		
DAVID				
Function	Gene Number	Enrichment	P Value	Benjamini
Stromal				
Signal	150 genes	8.34	2.3×10^{-13}	5.9×10^{-11}
Extracellular region	64	5.03	4.9×10^{-8}	4.6×10^{-6}
Cell adhesion	29	3.54	4.5×10^{-4}	7.4×10^{-2}
Vasculature development	17	2.66	1.4×10^{-5}	4.2×10^{-3}
EGF-like region	12	2.17	7.5×10^{-4}	1.5×10^{-1}
Neuropeptide signal	10	1.88	1.7×10^{-4}	4.1×10^{-2}
Carbohydrate binding	7	1.83	7.2×10^{-4}	1.2×10^{-1}
Hormone regulation	11	1.78	3.9×10^{-4}	7.4×10^{-2}
Epithelial				
Extracellular	213 genes	5.41	8.1×10^{-8}	1.9×10^{-5}
Signal	35	5.37	1.6×10^{-9}	5.6×10^{-7}
Vasculature development	48	2.44	1.1×10^{-3}	1.3×10^{-1}
Oxidoreductase	11	2.27	4.7×10^{-5}	3.3×10^{-3}

(Figure 4B). Conversely, *Aldo-Ketose Reductase family 1 member C1 (AKR1C1)* was upregulated by miR-200b mimic and downregulated by miR-200b antagomir. The miR-204 mimic and antagomir downregulated *PTCH1* and upregulated *CREB5* expression, respectively (Figure 4B).

Target Genes of miR-504 and miR-1827 in Epithelial Cells

Similarly, miR-504 and miR-1827 mimics and antagomirs were transiently transfected into diseased and normal epithelial cells, and qPCR confirmed their altered expression following modulation (Figure 4C). Selected predicted target genes of miR-504 and miR-1827 in the epithelial cell population were analyzed (Figure 4D). The miR-504 mimic downregulated *Neuropilin 2 (NRP2)* in endometriotic and normal epithelial cells. The miR-1827 mimic downregulated *ELM2* And *Myb/SANT domain containing*

1 (ELMSAN1) and *PLXNA4* in normal epithelial cells and *NAD(P)H Quinone dehydrogenase 1 (NQO1)* in endometriotic epithelial cells. Conversely, miR-1827 antagomir upregulated *ELMSAN1* in normal epithelial cells.

Discussion

This study demonstrates that endometriotic stromal and epithelial cells are associated with uniquely aberrant molecular pathways that were regulated by microRNAs. In endometriotic stromal cells, these associated aberrant pathways interfere with immune cell trafficking and impair decidualization through the Akt/ERK/P38MAPK/TNF/NF-κB pathway, and specific target gene members were modulated by miR-200b and miR-204. In contrast, dysregulation of the IL-1B/Akt/ERK/NF-κB/*Vascular Endothelial Growth Factor (VEGF)* and TGFβ1/BMP2/MMP2/COX2/ZEB1 pathways associated with endometriotic

Figure 4. A, miR-200b and miR-204 expression after transfection of mimics and antagomirs into endometriotic and normal stromal cells validated by quantitative polymerase chain reaction (qPCR), normalized with SNORD44. B, miR-200b and miR-204 target gene expression analyzed by qPCR for endometriotic compared to normal stromal cells, normalized with 18S RNA, $n = 4$. C, miR-504 and miR-1827 expression after transfection of mimics and antagomirs into endometriotic and normal epithelial cells validated by qPCR, normalized with SNORD44. D, miR-504 and miR-1827 target gene expression analyzed by qPCR for endometriotic compared to normal epithelial cells, normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), $n = 6$; Mean (standard deviation). $*P \leq .05$, $**P \leq .01$. antagomir Endo indicates antagomir endometriosis; antagomir N, antagomir normal; mimic N, mimic normal; mimic Endo, mimic endometriosis;

Figure 4. (continued) AKR1C1, Aldo-Keto Reductase family 1 member C1; CREB5, CAMP Responsive Element Binding Protein 5; ELMSAN1, ELM2 And Myb/SANT Domain containing 1; FOXF1, Forkhead box F1; NQO1, NAD(P)H Quinone dehydrogenase 1; NRP2, Neuropilin 2; PLXNA4, Plexin A4; PTCH1, Patched 1; ZEB1, Zinc Finger E-Box Binding homeobox 1.

epithelial cells may induce inflammation and potential activation of EMT, respectively (Figure 5A).

Impaired decidualization and aberrant immune cell trafficking of stromal cells in endometriosis often result in infertility or poor embryo implantation.⁶⁸⁻⁷⁰ Diseased stromal cells potentially modulate immune cell trafficking mainly by downregulation of the cytokines *IL-15*, *CXCL12* and the mediator of IL-1, *MYD88*, that decrease movement of mast and antigen-presenting dendritic and macrophage cells (Table 3).⁷¹⁻⁷³ Decreased immune cell trafficking has 2 consequences, first, reduced inflammatory response to infectious pathogens, such as bacteria,⁷⁴ and second, the reduced influx

of dendritic, uterine natural killer (uNK), mast, and macrophage cells leads to several reproductive disorders, such as embryo implantation failure, recurrent miscarriages, and pre-eclampsia.^{69,75} Interleukin 15 both responds to infectious pathogens and stimulates proliferation and differentiation of uNK cells in the endometrium during decidualization.^{76,77} Upon decidualization, dendritic and uNK cells crucially remodel endometrial spiral arteries ready for trophoblast invasion that will convert the arteries into wide-diameter, high-volume blood vessels for placental development.^{78,79} Decidual cells act as a physical barrier to prevent the excessive invasion of the columns of extravillous trophoblast

Figure 5. A, Transforming growth factor β 1 (TGF β 1)/ZEB1/miR-200b feedback loop for endometriotic epithelial cells in EMT. B, miR-200b/ZEB1 double-negative feedback loop for endometriotic stromal cells in MET. C, miR-1827/miR-504 model of target genes and pathways in endometriotic epithelial cells. BMP2 indicates Bone Morphogenetic Protein 2; COX2 (PTGS2), Prostaglandin-endoperoxide Synthase 2; DNMT1, deoxynucleotidyltransferase terminal interacting protein 1; ELMSAN1, (MIDEAS) ELM2 and Myb/SANT domain containing 1; EMT, epithelial–mesenchymal transition; FGF2, Fibroblast Growth Factor 2; FOXO, Forkhead Box; HDAC1, histone deacetylase 1; IL-1B, Interleukin 1B; KLF2/4, Kruppel Like Factor 2/4; MET, mesenchymal–epithelial transition; MMP2, Matrix Metalloproteinase 2; NQO1, NAD(P)H Quinone dehydrogenase 1; NRP2, neuropilin 2; OCT4 (POU5F1), POU class 5 homeobox 1; PLXNA4, plexin A4; SEMA, semaphorin; SOX2, SRY-box 2; MMP2, Matrix Metalloproteinase 2; TGF β 1, transforming growth factor beta 1; VEGF, Vascular Endothelial Growth Factor; ZEB1, Zinc finger E-box Binding protein 2.

cells.^{59,80,81} However, if decidualization is impaired, then trophoblasts will invade too deeply, and the life-threatening disorders of choriocarcinoma or placenta accreta may occur.^{58,60,82}

Key decidualization genes were found to be aberrantly expressed and may contribute to impaired decidualization in endometriotic stromal cells, such as upregulation of *POSTN*, *SERPINA1*, *MMP7*, and miR-200b and downregulation of *HAND2*, *IL-15*, *ZEB1*, and *WNT4* (Figure 2C).^{66,83,84} Although the stromal cells analyzed came from the proliferative phase, it has been demonstrated by other studies that aberrant expression of key decidualization genes in the proliferative phase, such as *POSTN*, *FK506 Binding Protein 4* (*FKBP4*), progesterone receptor chaperone, *Corticotropin Releasing Hormone* (*CRH*), and *Urocortin* (*UCN*), have persisted into the secretory phase and adversely affect decidualization by delaying the phase transition due to progesterone resistance of endometriosis.^{24,84–87} During decidualization, the miR-200 family

is downregulated; however, miR-200b is upregulated in endometriotic stromal cells during the proliferative phase (Figure 3C), potentially affecting decidualization by downregulating *CREB5*, *Sulfatase 2* (*SULF2*), and *ZEB1*, which are normally upregulated during decidualization.^{84,88} *CREB5* is a member of the important cyclic adenosine monophosphate/protein kinase A induction pathway of decidualization.⁸⁹ Impaired decidualization presages that the embryo will have difficulty implanting into the endometrium and, if successful, may result in poor placental development or miscarriage.^{90,91} A poorly developed placenta can lead to preeclampsia, preterm birth, or intrauterine growth restriction.^{82,92,93}

Interestingly, in endometriotic stromal cells, the downregulation of *ZEB1* through aberrant upregulation of miR200b (Figure 4B)^{94–97} potentially activates a mesenchymal–epithelial transition (MET) pathway by a double-negative feedback loop.^{98–100} Double-negative feedback loops consist of 2 molecular interactions that reciprocally repress each other, depending on their

relative levels, and act as a reversible molecular switch that can maintain bistable cellular states, for example, in this case, either stromal or epithelial cells, by switching between either EMT or MET depending on the prevailing molecular factors. Activation of MET has been demonstrated by Wang et al to mediate early-stage induction of fibroblast dedifferentiation into induced pluripotent stem cells (Figure 5B).¹⁰¹ It is not clear what properties of MET-transformed stromal cells contribute to endometriosis other than, perhaps, being prone to form lesions ectopically.¹⁰²

Progesterone-resistant dysregulation of decidualization has been shown to play a significant role in the pathogenesis of endometriosis.^{50,103–105} Increased *POSTN* upregulates Akt (protein kinase B), which downregulates the progesterone receptors (PRA/B), so that increased Akt activation in endometriosis inhibits decidualization.^{106,107} Decreased *HAND2* in endometriotic stromal cells, demonstrated here, may impact proper metabolism of estradiol and instead downregulate critical genes for decidualization of *IL-15*, *TIMP* *Metalloproteinase Inhibitor 3* (*TIMP3*), and *FOXO1*.¹⁰⁸ Progesterone resistance is also due to aberrant expression of steroid biosynthesis enzymes in endometriotic stromal and epithelial cells.^{63,105} The aldo-keto reductases *AKR1C1/2/3* are progesterone and steroid metabolizers that are upregulated in endometriosis.¹⁰⁹ Increased *AKR1C1* (20 α -hydroxysteroid dehydrogenase) inactivates progesterone, and in stromal cells, miR-200b overexpression upregulated *AKR1C1* (Figure 4B). *AKR1C2* inactivates potent 5- α -Dihydrotestosterone (5 α -DHT) and is upregulated in endometriotic epithelial cells (Table 1).¹⁰⁹ Other metabolizing enzymes, such as 17 β -hydroxysteroid dehydrogenase 2 (*HSD17B2*), metabolize estradiol to weakly estrogenic estrone and *HSD11B2*, which inactivates cortisol. *HSD17B2* and *HSD11B2* were both downregulated in endometriotic epithelial cells (Table 1).⁶³ *HSD17B2* in epithelial cells is regulated by stromal paracrine factors, especially retinoic acid, which in normal stromal cells is upregulated by progesterone.^{65,110} However, in endometriotic stromal cells, progesterone resistance disrupts the paracrine retinoic acid signaling and downregulates *HSD17B2* in epithelial cells so that estradiol is not properly metabolized and abnormally accumulates.^{63,65,106}

Progesterone resistance from stromal cells also disrupts the 2-way paracrine interaction between stromal and epithelial cells.^{64,111} Decreased progesterone signaling in the stromal cells downregulates Indian Hedgehog (*IHH*) in epithelial cells, which is downregulated in endometriosis.^{64,112,113} Not only is *IHH* downregulated in diseased epithelial cells with decreased signal to stromal cells, but also the Hedgehog receptor, *PTCH1*, in stromal cells, is downregulated by miR-200b and miR-204 (Figure 4B). Hedgehog signaling in stromal cells also involves *CREB5* and *FOXF1*, the target genes of miR-200b and miR-204, and *NRP2*, the target gene of miR-504 in epithelial cells.^{114,115} The Hedgehog pathway and miR-204 in endometriotic stromal cells separately downregulate *WNT4*, essential for decidualization.⁶⁶ So the dysregulated Hedgehog signaling pathway from endometriotic epithelial cells impairs decidualization in stromal cells.⁶²

Furthermore, in diseased epithelial cells, inflammation-related genes are upregulated, such as *TGF β 1*, *IL-1B*, *BMP2*, *PTGS2* (COX2), *SERPINA1*, and *CSF3*.¹¹⁶ (*SERPINA1* and *CSF3* are in common with diseased stromal cells as well as downregulated *LTBP4*, which releases increased TGF β 1.) Increased TGF β 1 expression activates the ZEB1 molecular pathway to EMT and may increase pluripotency and stemness (Figure 5A) as well as increase the homing of mononuclear leukocytes (Table 3).^{94,117–121} An increased expression of TGF β 1, MMP2, COX2, and BMP2 upregulates *ZEB1*.^{122–124} *ZEB1* overexpression promotes metastasis in cancer and marks aggressive endometrial carcinoma.^{117,125–127} *ZEB1* activates EMT by repressing E-cadherin (*CDH1*), which dissociates cell adhesion and disrupts epithelial cell polarity.⁹⁶ *ZEB1* represses stemness-inhibiting microRNAs, especially miR-200b, to induce stem cell-like properties in epithelial cells, notably in cancer, including endometrial carcinoma (Figure 5A).^{95,117,128,129} Although the downregulation of miR-200b was not discernible in this study of endometriotic epithelial cells, other studies have found that miR-200b is downregulated in endometriotic endometrium.^{18,21,130} Perhaps the role of miR-200b in the ZEB1/miR-200b feedback loop in endometriotic epithelial cells is for attachment at an ectopic site, so that upregulated miR-200b inhibits *ZEB1*, reverses EMT, and induces MET.¹²⁹ (Similarly, downregulated *ZEB1* in diseased stromal cells could activate an MET pathway in eutopic endometrium.) The EMT in endometriotic epithelial cells might also be activated by Hedgehog signaling indirectly via Fibroblast Growth Factor 2 (FGF2), NOTCH, and TGF β 1 signaling pathways or by TGF β 1, *ZEB1*, and miR-504 upregulating *NRP2* (Figures 3H and 5C).^{114,131} In turn, EMT strongly induces *AXL receptor tyrosine kinase* (*AXL*), which was upregulated in endometriotic epithelial cells in this study (Table 1). *AXL* increases cell migration and metastasis and is a stem cell regulator.^{132–136}

When potentially invasive, endometriotic, stem cell-like EMT cells are refluxed during menstruation and attach in ectopic sites, these EMT cells are ready primed by IL-1B for neovascularization with altered expression of *Nitric Oxide Synthase 3* (*NOS*), *FGF2*, *MMP2*, *TGF β 1*, *BMP2*, and *FLT1* to enable invasion.^{118,128} The target genes of miR-504 and miR-1827, *NRP2* and *NQO1*, increase vascularization, leukocyte, and cell migration in endometriotic epithelial cells (Table 3; Figure 5C). *NRP2* and *PLXNA4* are neovascularization pathfinders for ectopic sites.¹³⁷ However, ectopic lesions, compared to eutopic endometrium, can have reversed molecular expression of critical genes. Other studies have shown that when the refluxed EMT cells are implanted at an ectopic site, *ZEB1* is downregulated, E-cadherin and miR-200b are upregulated, the cells transform back into epithelial cells by MET, and these cells establish, vascularize, and grow into lesions.^{102,138}

In conclusion, aberrant molecular pathways associated with diseased stromal cells decrease immune cell trafficking and impair decidualization, while diseased epithelial cells are associated with TGF β 1 and IL-1B inflammation-driven pathways that increase vascularization, cell proliferation, leukocyte and cell

migration, and pathological EMT. The target genes of the differentially expressed microRNAs, such as miR-200b, miR-204, miR-504, and miR-1827, play a prominent role in the main aberrant functions associated with endometriotic stromal and epithelial cells. Understanding these cell type-specific microRNA—mRNA molecular pathways and interactions will contribute to the targeted treatment of this complex and devastating disease.

Acknowledgments

The authors thank Kim Chi Vo and Jacquelyn Hoffman, from the National Institutes of Health (NIH)/University of California, San Francisco (UCSF) Human Endometrial Tissue and DNA Bank, for collecting the endometrial tissue samples.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Institutes of Health (Grant number NIH-U54HD055764).

ORCID iD

Philip C. Logan, PhD <http://orcid.org/0000-0001-5656-6538>.

Supplemental Material

Supplementary material for this article is available online.

References

- Giudice LC. Endometriosis. *N Engl J Med*. 2010;362(25):2389-2398.
- Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. *Fertil Steril*. 2012;98(3):511-519.
- Agarwal N, Subramanian A. Endometriosis—morphology, clinical presentations and molecular pathology. *J Lab Physicians*. 2010;2(2):1-9.
- Brosens I, Puttemans P, Benagiano G. Endometriosis: a life cycle approach? *Am J Obstet Gynecol*. 2013;209(4):307-316.
- Brosens I, Brosens J, Benagiano G. Neonatal uterine bleeding as antecedent of pelvic endometriosis. *Hum Reprod*. 2013;28(11):2893-2897.
- Lessey B, Lebovic D, Taylor R. Eutopic endometrium in women with endometriosis: ground zero for the study of implantation defects. *Semin Reprod Med*. 2013;31(2):109-124.
- Hadfield R, Mardon H, Barlow D, Kennedy S. Delay in the diagnosis of endometriosis: a survey of women from the USA and the UK. *Hum Reprod*. 1996;11(4):878-880.
- Maruyama T, Yoshimura Y. Stem cell theory for the pathogenesis of endometriosis. *Front Biosci*. 2012;4E(8):2754-2763.
- Aznaurova Y, Zhumataev M, Roberts T, Aliper A, Zhavoronkov A. Molecular aspects of development and regulation of endometriosis. *Reprod Biol Endocrinol*. 2014;12(1):50.
- Gargett CE, Schwab KE, Brosens JJ, Puttemans P, Benagiano G, Brosens I. Potential role of endometrial stem/progenitor cells in the pathogenesis of early-onset endometriosis. *Mol Hum Reprod*. 2014;20(7):591-598.
- Du H, Taylor HS. Contribution of bone marrow-derived stem cells to endometrium and endometriosis. *Stem Cells*. 2007;25(8):2082-2086.
- Zanatta A, Rocha A, Carvalho F, et al. The role of the Hoxa10/HOXA10 gene in the etiology of endometriosis and its related infertility: a review. *J Assist Reprod Genet*. 2010;27(12):701-710.
- Sampson J. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol*. 1927;14(4):422-469.
- Marsh EE, Laufer MR. Endometriosis in premenarcheal girls who do not have an associated obstructive anomaly. *Fertil Steril*. 2005;83(3):758-760.
- Matsuura K, Ohtake H, Katabuchi H, Okamura H. Coelomic metaplasia theory of endometriosis: evidence from in vivo studies and an in vitro experimental model. *Gynecol Obstet Invest*. 1999;47(suppl 1):18-22.
- Figueira PGM, Abrão MS, Krikun G, Taylor H. Stem cells in endometrium and their role in the pathogenesis of endometriosis. *Ann NY Acad Sci*. 2011;1221(1):10-17.
- Wren JD, Wu Y, Guo SW. A system-wide analysis of differentially expressed genes in ectopic and eutopic endometrium. *Hum Reprod*. 2007;22(8):2093-2102.
- Ohlsson Teague EMC, Van der Hoek KH, Van der Hoek MB, et al. MicroRNA-regulated pathways associated with endometriosis. *Mol Endocrinol*. 2009;23(2):265-275.
- Matsuzaki S. DNA microarray analysis in endometriosis for development of more effective targeted therapies. *Front Biosci*. 2011;3E(3):1139-1153.
- Painter JN, Anderson CA, Nyholt DR, et al. Genome-wide association study identifies a locus at 7p15.2 associated with endometriosis. *Nat Genet*. 2011;43(1):51-54.
- Filigheddu N, Gregnanin I, Porporato PE, et al. Differential expression of microRNAs between eutopic and ectopic endometrium in ovarian endometriosis. *J Biomed Biotechnol*. 2010;2010:369549.
- Sun PR, Jia SZ, Lin H, Leng JH, Lang JH. Genome-wide profiling of long noncoding ribonucleic acid expression patterns in ovarian endometriosis by microarray. *Fertil Steril*. 2014;101(4):1038-1046.e7.
- Matsuzaki S, Canis M, Vaur-Barrière C, Boespflug-Tanguy O, Dastugue B, Mage G. DNA microarray analysis of gene expression in eutopic endometrium from patients with deep endometriosis using laser capture microdissection. *Fertil Steril*. 2005;84(suppl 2):1180-1190.
- Burney RO, Talbi S, Hamilton AE, et al. Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. *Endocrinology*. 2007;148(8):3814-3826.
- Graham A, Falcone T, Nothnick WB. The expression of microRNA-451 in human endometriotic lesions is inversely related to that of macrophage migration inhibitory factor (MIF) and regulates MIF expression and modulation of epithelial cell survival. *Hum Reprod*. 2015;30(3):642-652.
- Sherwin JRA, Sharkey AM, Mihalyi A, Simsa P, Catalano RD, D'Hooghe TM. Global gene analysis of late secretory phase, eutopic

- endometrium does not provide the basis for a minimally invasive test of endometriosis. *Hum Reprod.* 2008;23(5):1063-1068.
27. Meola J, Rosa e Silva JC, Dentillo DB, et al. Differentially expressed genes in eutopic and ectopic endometrium of women with endometriosis. *Fertil Steril.* 2010;93(6):1750-1773.
 28. Rosser MD, Haris PI, Ankrett DN, Konje JC. The emerging role of epigenetics and miRNAs in endometriosis. *Expert Rev Obstet Gynecol.* 2011;6(4):431-450.
 29. Kennedy SH. The genetics of endometriosis. *Eur J Obstet Gynecol Reprod Biol.* 1999;82(2):129-133.
 30. Guo SW. Epigenetics of endometriosis. *Mol Hum Reprod.* 2009;15(10):587-607.
 31. Baccarelli A, Bollati V. Epigenetics and environmental chemicals. *Curr Opin Pediatr.* 2009;21(2):243-251.
 32. Lee B, Du H, Taylor HS. Experimental murine endometriosis induces DNA methylation and altered gene expression in eutopic endometrium. *Biol Reprod.* 2009;80(1):79-85.
 33. Xue Q, Lin Z, Cheng YH, et al. Promoter methylation regulates estrogen receptor 2 in endometrium and endometriosis. *Biol Reprod.* 2007;77(4):681-687.
 34. Dyson MT, Roqueiro D, Monsivais D, et al. Genome-wide DNA methylation analysis predicts an epigenetic switch for GATA factor expression in endometriosis. *PLoS Genet.* 2014;10(3):e1004158.
 35. Wu Y, Strawn E, Basir Z, Halverson G, Guo SW. Aberrant expression of deoxyribonucleic acid methyltransferases DNMT1, DNMT3A, and DNMT3B in women with endometriosis. *Fertil Steril.* 2007;87(1):24-32.
 36. Nasu K, Kawano Y, Tsukamoto Y, et al. Aberrant DNA methylation status of endometriosis: epigenetics as the pathogenesis, biomarker and therapeutic target. *J Obstet Gynaecol Res.* 2011;37(7):683-695.
 37. Naqvi H, Ilagan Y, Krikun G, Taylor HS. Altered genome-wide methylation in endometriosis. *Reprod Sci.* 2014;21(10):1237-1243.
 38. Colon-Diaz M, Baez-Vega P, Garcia M, et al. HDAC1 and HDAC2 are differentially expressed in endometriosis. *Reprod Sci.* 2012;19(5):483-492.
 39. Kawano Y, Nasu K, Li H, et al. Application of the histone deacetylase inhibitors for the treatment of endometriosis: histone modifications as pathogenesis and novel therapeutic target. *Hum Reprod.* 2011;26(9):2486-2498.
 40. Nasu K, Kawano Y, Kai K, et al. Aberrant histone modification in endometriosis. *Front Biosci.* 2014;19:1202-1214.
 41. Monteiro JB, Colón-Díaz M, García M, et al. Endometriosis is characterized by a distinct pattern of histone 3 and histone 4 lysine modifications. *Reprod Sci.* 2014;21(3):305-318.
 42. Taft RJ, Pang KC, Mercer TR, Dinger M, Mattick JS. Non-coding RNAs: regulators of disease. *J Pathol.* 2010;220(2):126-139.
 43. Santamaria X, Taylor H. MicroRNA and gynecological reproductive diseases. *Fertil Steril.* 2014;101(6):1545-1551.
 44. Petracco R, Grechukhina O, Popkhadze S, Massasa E, Zhou Y, Taylor HS. MicroRNA 135 regulates HOXA10 expression in endometriosis. *J Clin Endocrinol Metab.* 2011;96(12):E1925-E1933.
 45. Ohlsson TE, Print CG, Hull ML. The role of microRNAs in endometriosis and associated reproductive conditions. *Hum Reprod Update.* 2010;16(2):142-165.
 46. Hawkins SM, Creighton CJ, Han DY, et al. Functional microRNA involved in endometriosis. *Mol Endocrinol.* 2011;25(5):821-832.
 47. Pan Q, Chegini N. MicroRNA signature and regulatory functions in the endometrium during normal and disease states. *Semin Reprod Med.* 2008;26(6):479-493.
 48. Braza-Boïls A, Mari-Alexandre J, Gilabert J, et al. MicroRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors. *Hum Reprod.* 2014;29(5):978-988.
 49. Okamoto M, Nasu K, Abe W, et al. Enhanced miR-210 expression promotes the pathogenesis of endometriosis through activation of signal transducer and activator of transcription 3. *Hum Reprod.* 2015;30(3):632-641.
 50. Kao LC, Germeyer A, Tulac S, et al. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. *Endocrinology.* 2003;144(7):2870-2881.
 51. Burney RO, Hamilton AE, Aghajanova L, et al. MicroRNA expression profiling of eutopic secretory endometrium in women with versus without endometriosis. *Mol Hum Reprod.* 2009;15(10):625-631.
 52. Zafrakas M, Tarlatzis BC, Streichert T, et al. Genome-wide microarray gene expression, array-CGH analysis, and telomerase activity in advanced ovarian endometriosis: a high degree of differentiation rather than malignant potential. *Int J Mol Med.* 2008;21(3):335-344.
 53. Oger F, Gheeraert C, Mogilenko D, et al. Cell-specific dysregulation of MicroRNA expression in obese white adipose tissue. *J Clin Endocrinol Metab.* 2014;99(8):2821-2833.
 54. Jovićić A, Roshan R, Moiso N, et al. Comprehensive expression analyses of neural cell-type-specific miRNAs identify new determinants of the specification and maintenance of neuronal phenotypes. *J Neurosci.* 2013;33(12):5127-5137.
 55. McCall MN, Kent OA, Yu J, Fox-Talbot K, Zaiman AL, Halushka MK. MicroRNA profiling of diverse endothelial cell types. *BMC Med Genomics.* 2011;4(1):1-13.
 56. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med.* 2007;25(6):445-453.
 57. Guzeloglu-Kayisli O, Kayisli UA, Taylor HS. The role of growth factors and cytokines during implantation: endocrine and paracrine interactions. *Semin Reprod Med.* 2009;27(1):62-79.
 58. Graham C, Lala P. Mechanisms of placental invasion of the uterus and their control. *Biochem Cell Biol.* 1992;70(10-11):867-874.
 59. Lockwood C, Krikun G, Schatz F. The decidua regulates hemostasis in human endometrium. *Semin Reprod Endocrinol.* 1999;17(1):45-51.
 60. Norwitz ER. Defective implantation and placentation: laying the blueprint for pregnancy complications. *Reprod Biomed Online.* 2006;13(4):591-599.
 61. Aplin JD. Embryo implantation: the molecular mechanism remains elusive. *Reprod Biomed Online.* 2006;13(6):833-839.
 62. Kurihara I, Lee DK, Petit FG, et al. COUP-TFII mediates progesterone regulation of uterine implantation by controlling ER activity. *PLoS Genetics.* 2007;3(6):e102.

63. Bulun SE, Cheng YH, Pavone ME, et al. 17 β -hydroxysteroid dehydrogenase-2 deficiency and progesterone resistance in endometriosis. *Semin Reprod Med.* 2010;28(1):44-50.
64. Lee KY, Jeong JW, Tsai SY, Lydon JP, DeMayo FJ. Mouse models of implantation. *Trend Endocrinol Metab.* 2007;18(6):234-239.
65. Bulun SE. Endometriosis. *N Engl J Med.* 2009;360(3):268-279.
66. Li Q, Kannan A, Das A, et al. WNT4 acts downstream of BMP2 and functions via β -catenin signaling pathway to regulate human endometrial stromal cell differentiation. *Endocrinology.* 2013;154(1):446-457.
67. Gregory GD, Vakoc CR, Rozovskaia T, et al. Mammalian ASH1L is a histone methyltransferase that occupies the transcribed region of active genes. *Mol Cell Biol.* 2007;27(24):8466-8479.
68. Klemmt PA, Carver JG, Kennedy SH, Koninckx PR, Mardon HJ. Stromal cells from endometriotic lesions and endometrium from women with endometriosis have reduced decidualization capacity. *Fertil Steril.* 2006;85(3):564-572.
69. Lash GE, Bulmer JN. Do uterine natural killer (uNK) cells contribute to female reproductive disorders? *J Reprod Immunol.* 2011;88(2):156-164.
70. Salamonsen L, Hannan N, Dimitriadis E. Cytokines and chemokines during human embryo implantation: roles in implantation and early placentation. *Semin Reprod Med.* 2007;25(6):437-444.
71. Waldmann TA, Tagaya Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Ann Rev Immunol.* 1999;17(1):19-49.
72. Sozzani S, Allavena P, Vecchi A, Mantovani A. The role of chemokines in the regulation of dendritic cell trafficking. *J Leuk Biol.* 1999;66(1):1-9.
73. Schiraldi M, Raucci A, Muñoz LM, et al. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. *J Exp Med.* 2012;209(3):551-563.
74. Kalesnikoff J, Galli SJ. New developments in mast cell biology. *Nat Immunol.* 2008;9(11):1215-1223.
75. Thiruchelvam U, Dransfield I, Saunders PTK, Critchley HOD. The importance of the macrophage within the human endometrium. *J Leukoc Biol.* 2013;93(2):217-225.
76. Okada H, Nakajima T, Sanesumi M, Ikuta A, Yasuda K, Kanzaki H. Progesterone enhances interleukin-15 production in human endometrial stromal cells in vitro. *J Clin Endocrinol Metab.* 2000;85(12):4765-4770.
77. Godbole G, Modi D. Regulation of decidualization, interleukin-11 and interleukin-15 by homeobox A 10 in endometrial stromal cells. *J Reprod Immunol.* 2010;85(2):130-139.
78. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. *Clin Exp Reprod Med.* 2011;38(3):119-125.
79. Cartwright JE, Balarajah G. Trophoblast interactions with endothelial cells are increased by interleukin-1[β] and tumour necrosis factor [α] and involve vascular cell adhesion molecule-1 and [α]₄ β 1. *Exp Cell Res.* 2005;304(1):328-336.
80. Gellersen B, Reimann K, Samalecos A, Aupers S, Bamberger AM. Invasiveness of human endometrial stromal cells is promoted by decidualization and by trophoblast-derived signals. *Hum Reprod.* 2010;25(4):862-873.
81. Fisher SJ, Damsky CH. Human cytotrophoblast invasion. *Semin Cell Biol.* 1993;4(3):183-188.
82. Brosens I, Pijnenborg R, Vercruysse L, Romero R. The Great Obstetrical Syndromes are associated with disorders of deep placentation. *Am J Obstet Gynecol.* 2011;204(3):193-201.
83. Duncan WC, Shaw JLV, Burgess S, McDonald SE, Critchley HOD, Horne AW. Ectopic pregnancy as a model to identify endometrial genes and signaling pathways important in decidualization and regulated by local trophoblast. *PLoS One.* 2011;6(8):e23595.
84. Aghajanova L, Tatsumi K, Horcujadas JA, et al. Unique transcriptome, pathways, and networks in the human endometrial fibroblast response to progesterone in endometriosis. *Biol Reprod.* 2011;84(4):801-815.
85. Novembri R, Borges LE, Carrarelli P, et al. Impaired CRH and urocortin expression and function in eutopic endometrium of women with endometriosis. *J Clin Endocrinol Metab.* 2011;96(4):1145-1150.
86. Shen L, Liu P, Zhang P, Zhang X, Cui J. Characterization of periostin expression in human endometrium and endometriotic lesions. *Gynecol Endocrinol.* 2012;28(10):815-818.
87. Yang H, Zhou Y, Edelshtain B, Schatz F, Lockwood CJ, Taylor HS. FKBP4 is regulated by HOXA10 during decidualization and in endometriosis. *Reproduction.* 2012;143(4):531-538.
88. Estella C, Herrer I, Moreno-Moya JM, et al. miRNA signature and Dicer requirement during human endometrial stromal decidualization in vitro. *PLoS One.* 2012;7(7):e41080.
89. Gellersen B, Kempf R, Telgmann R. Human endometrial stromal cells express novel isoforms of the transcriptional modulator CREM and up-regulate ICER in the course of decidualization. *Mol Endocrinol.* 1997;11(1):97-113.
90. Teklenburg G, Salker M, Heijnen CJ, Macklon NS, Brosens JJ. The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. *Mol Hum Reprod.* 2010;16(12):886-895.
91. Lockwood C, Krikun G, Rahman M, Caze R, Buchwalder L, Schatz F. The role of decidualization in regulating endometrial hemostasis during the menstrual cycle, gestation, and in pathological states. *Semin Thromb Hemost.* 2007(01):111-117.
92. Kaufmann P, Black S, Huppertz B. Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. *Biol Reprod.* 2003;69(1):1-7.
93. Lucas ES, Dyer NP, Murakami K, et al. Loss of endometrial plasticity in recurrent pregnancy loss. *Stem cells.* 2016;34(2):346-356.
94. Brabletz S, Brabletz T. The ZEB/miR-200 feedback loop—a motor of cellular plasticity in development and cancer? *EMBO Rep.* 2010;11(9):670-677.
95. Renthal NE, Chen CC, Williams KC, Gerard RD, Prange-Kiel J, Mendelson CR. miR-200 family and targets, ZEB1 and ZEB2, modulate uterine quiescence and contractility during pregnancy and labor. *Proc Natl Acad Sci.* 2010;107(48):20828-20833.

96. Romero-Perez L, Lopez-Garcia MA, Diaz-Martin J, et al. ZEB1 overexpression associated with E-cadherin and microRNA-200 downregulation is characteristic of undifferentiated endometrial carcinoma. *Mod Pathol*. 2013;26(11):1514-1524.
97. Haraguchi H, Saito-Fujita T, Hirota Y, et al. MicroRNA-200a locally attenuates progesterone signaling in the cervix, preventing embryo implantation. *Mol Endocrinol*. 2014;28(7):1108-1117.
98. Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol*. 2008;10(5):593-601.
99. Hill L, Browne G, Tulchinsky E. ZEB/miR-200 feedback loop: at the crossroads of signal transduction in cancer. *Int J Cancer*. 2013;132(4):745-754.
100. Dykxhoorn DM. MicroRNAs and metastasis: little RNAs go a long way. *Cancer Res*. 2010;70(16):6401-6406.
101. Wang G, Guo X, Hong W, et al. Critical regulation of miR-200/ZEB2 pathway in Oct4/Sox2-induced mesenchymal-to-epithelial transition and induced pluripotent stem cell generation. *Proc Natl Acad Sci*. 2013;110(8):2858-2863.
102. Yao D, Dai C, Peng S. Mechanism of the mesenchymal-epithelial transition and its relationship with metastatic tumor formation. *Mol Cancer Res*. 2011;9(12):1608-1620.
103. Barragan F, Irwin JC, Balayan S, et al. Human endometrial fibroblasts derived from mesenchymal progenitors inherit progesterone resistance and acquire an inflammatory phenotype in the endometrial niche in endometriosis. *Biol Reprod*. 2016;94(5):118, 1-20.
104. Bulun SE, Cheng YH, Pavone ME, et al. Estrogen receptor-beta, estrogen receptor-alpha, and progesterone resistance in endometriosis. *Semin Reprod Med*. 2010;28(1):36-43.
105. Bulun SE, Cheng YH, Yin P, et al. Progesterone resistance in endometriosis: link to failure to metabolize estradiol. *Mol Cell Endocrinol*. 2006;248(1-2):94-103.
106. Eaton JL, Unno K, Caraveo M, Lu Z, Kim JJ. Increased AKT or MEK1/2 activity influences progesterone receptor levels and localization in endometriosis. *J Clin Endocrinol Metab*. 2013;98(12):e1871-e1879.
107. Morra L, Moch H. Periostin expression and epithelial-mesenchymal transition in cancer: a review and an update. *Virchows Arch*. 2011;459(5):465-475.
108. Shindoh H, Okada H, Tsuzuki T, Nishigaki A, Kanzaki H. Requirement of heart and neural crest derivatives-expressed transcript 2 during decidualization of human endometrial stromal cells in vitro. *Fertil Steril*. 2014;101(6):1781-1790.e5.
109. Hevir N, Vouk K, Šinkovec J, Ribič-Pucelj M, Rižner TL. Aldo-keto reductases AKR1C1, AKR1C2 and AKR1C3 may enhance progesterone metabolism in ovarian endometriosis. *Chem Biol Interact*. 2011;191(1-3):217-226.
110. Cheng YH, Utsunomiya H, Pavone ME, Yin P, Bulun SE. Retinoic acid inhibits endometrial cancer cell growth via multiple genomic mechanisms. *J Mol Endocrinol*. 2011;46(2):139-153.
111. Wetendorf M, DeMayo FJ. The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signaling network. *Mol Cell Endocrinol*. 2011;357(1-2):108-118.
112. Smith K, Alnifaidy R, Wei Q, Nieman LK. Endometrial Indian Hedgehog expression is decreased in women with endometriosis. *Fertil Steril*. 2011;95(8):2738-2741.e3.
113. Large MJ, DeMayo FJ. The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling. *Mol Cell Endocrinol*. 2012;358(2):155-165.
114. Katoh Y, Katoh M. Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (review). *Int J Mol Med*. 2008;22(3):271-275.
115. Hillman RT, Feng BY, Ni J, et al. Neuropilins are positive regulators of Hedgehog signal transduction. *Genes Dev*. 2011;25(22):2333-2346.
116. Fu L, Lockhart MG, Amir MM, Winnall WR, Rogers PAW, Girling JE. Differential TGFβ1-signalling in endometrium from women with endometriosis: importance of appropriate house-keeping genes. *J Endometr Pelvic Pain Disord*. 2014;6(1):41-54.
117. Gregory PA, Bracken CP, Smith E, et al. An autocrine TGF-β/ZEB/miR-200 signaling network regulates establishment and maintenance of epithelial-mesenchymal transition. *Mol Biol Cell*. 2011;22(10):1686-1698.
118. Proestling K, Birner P, Gamperl S, et al. Enhanced epithelial to mesenchymal transition (EMT) and upregulated MYC in ectopic lesions contribute independently to endometriosis. *Reprod Biol Endocrinol*. 2015;13(1):75.
119. Bartley J, Jülicher A, Hotz B, Mechsner S, Hotz H. Epithelial to mesenchymal transition (EMT) seems to be regulated differently in endometriosis and the endometrium. *Arch Gynecol Obstet*. 2013;289(4):871-881.
120. Fuxe J, Karlsson MCI. TGF-β-induced epithelial-mesenchymal transition: a link between cancer and inflammation. *Semin Cancer Biol*. 2012;22(5-6):455-461.
121. Moustakas A, Heldin CH. Induction of epithelial-mesenchymal transition by transforming growth factor β. *Semin Cancer Biol*. 2012;22(5-6):446-454.
122. Hurt E, Saykally J, Anose B, Kalli K, Sanders M. Expression of the ZEB1 (δEF1) transcription factor in human: additional insights. *Mol Cell Biochem*. 2008;318(1-2):89-99.
123. Abe W, Nasu K, Nakada C, Kawano Y, Moriyama M, Narahara H. miR-196b targets c-myc and Bcl-2 expression, inhibits proliferation and induces apoptosis in endometriotic stromal cells. *Hum Reprod*. 2013;28(3):750-761.
124. Anose BM, Sanders MM. Androgen receptor regulates transcription of the ZEB1 transcription factor. *Int J Endocrinol*. 2011;2011:903918.
125. Singh M, Spoelstra NS, Jean A, et al. ZEB1 expression in type I vs type II endometrial cancers: a marker of aggressive disease. *Mod Pathol*. 2008;21(7):912-923.
126. Gregory PA, Bracken CP, Bert AG, Goodall GJ. MicroRNAs as regulators of epithelial-mesenchymal transition. *Cell Cycle*. 2008;7(20):3112-3117.
127. Zhang GJ, Zhou T, Tian HP, Liu ZL, Xia SS. High expression of ZEB1 correlates with liver metastasis and poor prognosis in colorectal cancer. *Oncol Lett*. 2013;5(2):564-568.
128. Lebovic DI, Baldocchi RA, Mueller MD, Taylor RN. Altered expression of a cell-cycle suppressor gene, Tob-1, in endometriotic cells by cDNA array analyses. *Fertil Steril*. 2002;78(4):849-854.

129. Wellner U, Schubert J, Burk UC, et al. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat Cell Biol.* 2009;11(12):1487-1495.
130. Eggers JC, Martino V, Reinbold R, et al. microRNA miR-200b affects proliferation, invasiveness and stemness of endometriotic cells by targeting ZEB1, ZEB2 and KLF4. *Reprod BioMed Online.* 2016;32(4):434-445.
131. Nasarre P, Gemmill RM, Potiron VA, et al. Neuropilin-2 is upregulated in lung cancer cells during TGF β 1-induced epithelial-mesenchymal transition. *Cancer Res.* 2013;73(23):7111-7121.
132. Axelrod H, Pienta KJ. Axl as a mediator of cellular growth and survival. *Oncotarget.* 2014;5(19):8818-8852.
133. Asiedu MK, Beauchamp-Perez FD, Ingle JN, Behrens MD, Radisky DC, Knutson KL. AXL induces epithelial-to-mesenchymal transition and regulates the function of breast cancer stem cells. *Oncogene.* 2014;33(10):1316-1324.
134. Gjerdrum C, Tiron C, Høiby T, et al. Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. *Proc Natl Acad Sci.* 2010;107(3):1124-1129.
135. Sun WS, Misao R, Iwagaki S, Fujimoto J, Tamaya T. Coexpression of growth arrest-specific gene 6 and receptor tyrosine kinases, Axl and Sky, in human uterine endometrium and ovarian endometriosis. *Mol Hum Reprod.* 2002;8(6):552-558.
136. Honda H, Barreto FF, Gogusev J, Im DD, Morin PJ. Serial analysis of gene expression reveals differential expression between endometriosis and normal endometrium. Possible roles for AXL and SHC1 in the pathogenesis of endometriosis. *Reprod Biol Endocrinol.* 2008;6(1):1-13.
137. Torres-Vázquez J, Gitler AD, Fraser SD, et al. Semaphorinplexin signaling guides patterning of the developing vasculature. *Dev Cell.* 2004;7(1):117-123.
138. Saare M, Rekker K, Laisk-Podar T, et al. High-throughput sequencing approach uncovers the miRNome of peritoneal endometriotic lesions and adjacent healthy tissues. *PLoS One.* 2014;9(11):e112630.