Invited Review

MicroRNAs in endometriosis: biological function and emerging biomarker candidates[†]

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Abstract

MicroRNAs (miRNAs), a class of small noncoding RNA molecules, have been recognized as key post-transcriptional regulators associated with a multitude of human diseases. Global expression profiling studies have uncovered hundreds of miRNAs that are dysregulated in several diseases, and yielded many candidate biomarkers. This review will focus on miRNAs in endometriosis, a common chronic disease affecting nearly 10% of reproductive-aged women, which can cause pelvic pain, infertility, and a myriad of other symptoms. Endometriosis has delayed time to diagnosis when compared to other chronic diseases, as there is no current accurate, easily accessible, and noninvasive tool for diagnosis. Specific miRNAs have been identified as potential biomarkers for this disease in multiple studies. These and other miRNAs have been linked to target genes and functional pathways in disease-specific pathophysiology. Highlighting investigations into the roles of tissue and circulating miRNAs in endometriosis, published through June 2018, this review summarizes new connections between miRNA expression and the pathophysiology of endometriosis, including impacts on fertility. Future applications of miRNA biomarkers for precision medicine in diagnosing and managing endometriosis treatment are also discussed.

Summary Sentence

MicroRNAs exhibit altered abundance in women with endometriosis, are involved in the pathophysiology of the disease, and represent promising biomarkers.

Key words: endometriosis, microRNA, biomarker, diagnostic, infertility.

Introduction

Noncoding RNA molecules are increasingly recognized to play key roles in fine-tuning gene expression in human health and disease pathophysiology. Among these noncoding RNAs are microRNAs (miRNAs), which are short (~22 nucleotide) sequences that bind to complementary sequences of target mRNAs, to block translation or impact messenger RNA (mRNA) stability/degradation. First described as regulators of larval development in *Caenorhabditis elegans* [1, 2], miRNAs, such as those in the let-7 family, were soon recognized to be phylogenetically conserved across animal lineages [2]. They have since been appreciated as a widespread molecular mechanism for post-transcriptional regulation [3, 4]. Additionally, each

individual miRNA molecule may affect numerous mRNA targets across many cellular pathways [5]. More than 2600 mature miRNAs have been described in humans (miRBase.org updated March 2018, Release 22) [6].

MicroRNAs are found both intracellularly and in circulation; in fact, they have been found in most body fluids with unexpectedly high stability [7, 8]. Circulating miRNA expression profiles have been shown to differ significantly between healthy and diseased people [9]. Lawrie and colleagues were the first to describe circulating miRNAs in serum as potential noninvasive diagnostic markers for diffuse large B-cell lymphoma [10]. Since that time, changes in expression levels of specific miRNAs have been observed in a variety

of disease states including oncologic, inflammatory, cardiovascular, metabolic, and reproductive disorders. MicroRNAs are now proposed as clinical diagnostic tools for identifying and monitoring various cancers, with the potential to provide noninvasive diagnosis for many other clinical disorders [11]. Dysregulated miRNA expression has been explored in diseases of the female reproductive system, including gynecologic cancers (cervical, ovarian, and endometrial) [12], uterine leiomyomata [13], adenocarcinomas [14], pregnancy disorders such as preeclampsia and preterm birth [15, 16], and endometriosis [17].

Endometriosis is a benign inflammatory disease that occurs in up to 10% of reproductive-aged women [18]. The disease is defined by the presence of endometrial glands and stroma outside of the uterus, most commonly in the pelvic peritoneum, ovaries, anterior/posterior cul-de-sac, broad or uterosacral ligaments, as well as the sigmoid colon and appendix. Rarely, these estrogen-dependent ectopic implants have been found in the upper abdomen, lungs, diaphragm, and central nervous system [19]. Clinical manifestations vary, but can include dysmenorrhea, dyspareunia, chronic pelvic pain, constipation, dyschezia, urinary symptoms, and infertility. In contrast, some patients are asymptomatic or have only mild symptoms. This disease also has a familial tendency; compared to women with no family history of endometriosis, a woman with an affected first-degree relative has a seven times higher risk of developing the disease [20].

The exact pathogenesis of endometriosis remains elusive, but a number of theories have been proposed including retrograde menstruation, altered immunity, coelomic metaplasia, and metastatic spread, as well as possible stem cell and genetic origins. The most widely accepted theory was proposed by Sampson in 1927. In Sampson's theory of retrograde menstruation, endometrial tissue is sloughed in a retrograde fashion through the fallopian tubes during menses into the peritoneal cavity. Here, the cells attach to peritoneal mesothelial cells, establishing a blood supply. Cells then proliferate and produce endometrial implants, which continue to grow in response to estrogen signaling. Although 90% of women have evidence of retrograde menstruation, only 10% have the disease, suggesting that women with endometriosis have altered immunity that prevents them from clearing the refluxed endometrial tissue [21]. Cell-mediated immunity may be deficient, as leukocytes do not recognize that the ectopic endometrial tissue is foreign [22] and defective NK-cell activity has been shown to decrease cytotoxicity to endometrial cells [23]. The immune system also seems to potentiate the development and severity of disease once endometriosis has developed. Increased numbers of leukocytes and macrophages are found near endometrial implants and in peritoneal fluid, and these cells secrete cytokines and growth factors (IL-1, IL-6, IL-8, TNF, RANTES, VEGF) which leads to proliferation of implants [24–27]. Another theory, coelomic metaplasia, suggests that the peritoneum contains undifferentiated cells that can differentiate into endometrial cells [28]. Newer research suggests that stem cells may also play a role in the pathogenesis of endometriosis. In the last 15 years, multiple studies have shown that bone-marrow-derived cells may also differentiate into endometrial cells, thus explaining how ectopic tissue can occur in locations outside the peritoneal cavity [29–31].

One of the greatest challenges in endometriosis, to both patients and clinicians, is diagnosis, with a reported diagnostic delay of 6–12 years [32–34]. This delay is due to both societal and clinical factors. Women reportedly delay seeking care for symptoms of endometriosis because of embarrassment discussing menstrual irregularities/pain, a risk of stigmatization, and a feeling that their health care provider

did not take their symptoms seriously [33, 35, 36]. The delay of diagnosis is greater in women with more pelvic symptoms (chronic pelvic pain, dysmenorrhea, dyspareunia) and in women with a higher body mass index [32]. Despite years of chronic pelvic pain, discomfort, and other incapacitating symptoms, some women receive their first diagnosis of endometriosis when attempting, and failing, to conceive. Between 25 and 50% of women with infertility have endometriosis [19]. As detailed above, many of the symptoms of endometriosis can be nonspecific and overlap with other pelvic disorders. This overlap requires an increased level of clinical suspicion and awareness by providers to deliver a timely diagnosis. Notably, time to diagnosis is longer among women seeking care in a primary care setting, and also in centers with predominantly state-funded health care [32]. These settings often have an inherent disadvantage due to lack of access to clinical expertise and the lack of a current easily accessible and noninvasive diagnostic test suitable for screening. The gold standard for diagnosis of endometriosis is laparoscopy, which requires an operative procedure under general anesthesia. This can serve as a major barrier to primary care providers, and a cause for hesitancy among patients.

Prior to seeking the definitive diagnosis with laparoscopy, many patients and clinicians will begin treatment with a presumed diagnosis based on symptoms. First-line therapies for endometriosis include nonsteroidal anti-inflammatory drugs (NSAIDs) and combined hormonal contraceptives. Other medical treatment options include progestins, danazol, and gonadotropin-releasing hormone agonists (GnRH-a) [37]. Some of these therapies, however, are associated with significant side effects and typically do not provide long-term relief [38, 39]. Inferred diagnosis of endometriosis without laparoscopy is strengthened by an improvement in symptoms; however, biomarkers have been lacking and surgery has been considered the gold standard for definitive diagnosis. Laparoscopy provides the benefit of being both diagnostic and therapeutic; it continues to be a leading treatment for endometriosis that leads to improvement in pain. However, recurrence of endometriosis is common, with 50% of women requiring repeat surgery after 5 years [40].

The need exists for an accurate diagnostic method that is more easily accessible and noninvasive to allow for earlier diagnosis and initiation of medical therapies, as well as the ability to monitor the recurrence of the disease without using laparoscopy. Over the past 5–10 years, a substantial body of work has shed light on miRNAs as potentially robust biomarkers for endometriosis. Some of these dysregulated miRNAs show direct involvement in disease pathways, while others are associated specifically with the presence of the disease, but their biologic role is yet unknown. This review synthesizes knowledge of this emerging field, with particular emphasis on the miRNAs shown to be dysregulated both in circulation and in tissues, highlighting those that have been linked experimentally to biological processes of endometriosis. Profiling studies and functional studies (published through June 2018) of specific miRNAs and their gene targets were identified by searching the Pubmed.gov database for the keywords “endometriosis” and “miR” or “microRNA.”

Endometrial tissue miRNA profiling studies

The global profiling approach to identify miRNAs involved in the pathology of endometriosis compares the expression of all miRNAs between tissues. Most commonly this entails comparing the ectopic vs. eutopic endometrium (endometrial tissue lining the inside of the uterus) from the same patient, but some studies have profiled the eutopic endometrium from endometriosis patients vs. healthy controls.

MicroRNA microarrays and RNA sequencing are standard experimental approaches for capturing global miRNA expression. Candidate molecules are then selected based on statistically significant changes that reach a threshold of up- or downregulation (i.e. 2-fold, 10-fold). Typically, the differences in levels of candidate miRNAs are confirmed by techniques such as quantitative real-time polymerase chain reaction (qRT-PCR). Even small differences in miRNA levels may have biologically relevant consequences; thus, a large magnitude of up- or downregulation is not necessarily needed to observe a biological effect.

Nearly 20 such expression studies in endometrial tissues have been published in the last decade. Wei and colleagues conducted a meta-analysis of 12 miRNA expression profiling studies published between 2007 and 2013, reporting a total of 134 differentially regulated miRNAs, and 28 detected in two or more studies [41]. Since 2014, at least six other expression screens have reported findings from miRNA arrays or deep sequencing of endometrial lesions [42–44] or endometriomas vs. eutopic endometrium [45], or eutopic endometrium in endometriosis vs. controls [46]. In a recent review, Saare and colleagues highlight the tissue heterogeneity of the samples used to compare endometriosis to controls, categorizing studies as (1) lesion biopsy tissue vs. eutopic endometrial tissue, (2) endometrial tissue from endometriosis patients vs. controls, and (3) purified cell fractions from lesions vs. endometrial tissue [47]. Haikalis and colleagues quantified levels of six miRNAs in different types of endometriosis lesions (endometriomas, peritoneal, and deep infiltrating lesions), and reported expression profiles that were characteristic to each lesion type [48].

While individual studies generally report their own set of up- or downregulated miRNAs, the collection of miRNA molecules identified across multiple studies is growing, and miRNA regulatory networks are beginning to emerge [45]. The most frequently identified miRNA, found to be downregulated in six expression studies of endometriosis, was miR-200b [47], found in biopsy samples from endometriomas [42, 49, 50] and endometriotic lesions [43, 44, 51]; it is known to play a functional role in the epithelial-to-mesenchymal transition (EMT), an important process in endometriosis [52, 53]. Integration analysis of differentially expressed miRNAs, transcription factors, and mRNAs between endometriomas and eutopic endometrium also identified miR-200 family miRNAs, among other key regulatory miRNAs related to disease pathogenesis [45]. Interestingly, the miR-200 miRNA family is also important to the metastasis of ovarian cancer [54].

The following miRNAs were reported to be up- or downregulated in endometriotic lesions by three or more research studies: miR-1, -29c, -34c, -100, -141, -145, -183, -196b, -200a, -200b, -200c, -202, -365, and -375 [47]. Several of these are also known to be involved in EMT, as well as angiogenesis, cell proliferation, cell adhesion, and invasion [47, 55]. Both EMT and angiogenesis (development of new blood vessels) are key pathophysiological components in the establishment of endometriotic lesions. EMT is involved in cell migration and invasion during lesion formation, and newly established lesions require a blood supply to support their growth. Functional roles of a collection of dysregulated miRNAs in endometriotic lesions, partially overlapping with the list above, are discussed by Nothnick: miR-15, -20a, -23a/b, -29c, -126, -142, -145, -183, -199a, and -451 [56]. Distinctions are proposed between those miRNAs likely to be driving factors in the disease (regulators of cell proliferation, invasion, and angiogenesis) and the miRNAs for which expression changes occur as a downstream result of endometriosis pathology [56].

Individual miRNAs are known to regulate many different mRNA targets in cells, and these mRNAs and their encoded proteins may be linked by a common cellular pathway. To contextualize the mechanisms by which up- or downregulated miRNAs impact cell physiology, there are a myriad of bioinformatics tools and computational algorithms available to identify the predicted targets of each miRNA sequence, and map the function of those target genes onto cellular pathways and processes. In their systematic review, Wei et al. used four miRNA target prediction tools—TargetScan, PicTar, miRanda, and miRDB—on the miRNAs found consistently across several studies, and then predicted the biological processes and pathways these genes are involved in by means of the GeneCodis web tool [41]. A total of 9882 predicted miRNA target genes were identified, and the KEGG (Kyoto Encyclopedia for Genes and Genomes) and Panther pathways enrichment analysis yielded predominantly cancer-related pathways, endocytosis, Wnt signaling pathway, and angiogenesis [41].

Filigheddu et al. predicted the target mRNAs of their set of differentially expressed miRNAs with TARGETSCAN and PICTARVERT, arriving at over 3000 predicted targets, whose functions were then mapped and molecular pathways identified using Ingenuity Pathways Analysis software (Ingenuity IPA 7.5) [49]. Notably, they found that molecular networks corresponding to biological functions associated with endometriosis (cellular growth and proliferation, cell cycle, cell movement, and reproductive system disorders) were well represented in IPA results, and the reproductive disorder subcategory of “endometriosis” was detected as highly significant [49]. The molecular network converging on estrogen receptor 1 (ESR1) includes DNA methyltransferases that are validated targets of miR-29b, -29c, and -148a, and the authors cite the potential of aberrant methylation of HOXA10 and progesterone receptor PR-B to impact the altered expression of these genes in endometriosis [49]. Our research has shown that DNA methylation of HOXA10 may be a common mechanism in gynecological disease, with HOXA10 highly methylated at CpG sites in women with uterine polyps and myomas, and hypomethylated in women with endometriosis [57].

Other pathways identified in analysis of predicted targets of aberrantly expressed miRNAs were c-Jun, CREB-binding protein, protein kinase B (Akt), and cyclin D1 (CCND1) signaling [51]. In a study comparing endometriosis to ovarian cancer tissues, IPA revealed miRNA targets mapping to pathways of cell growth and development, cell migration, cell cycle, and cell death [55]. Recent work by Zhao and colleagues analyzed expression profiles of miRNA and mRNA in paired tissue samples of eutopic endometrium and endometriomas [45]. Using correlation analysis and bioinformatics, miR-transcription factor networks were identified, with five miRNA regulatory clusters including the miR-200 family, having predicted interactions with six families of transcription factors [45]. These bioinformatics analyses continue to inform experimental design for functional studies in cell culture, and can help identify new therapeutic targets. Directly demonstrating binding to target mRNAs *in vitro* is an important step to validate the function of candidate miRNAs, and functional experiments of miRNAs and specific mRNA targets in endometriosis are discussed below (see “Functional Studies” section and Supplemental Table S2, [136–159]).

Circulating miRNA profiling studies

Extracellular miRNAs can be isolated from most body fluids, including the circulation [7]. They are known to be involved in stress signaling and communication between cells and tissues in the body

Table 1. Global Expression Profiling studies of Circulating miRNAs in Endometriosis

Sample type	Control group	Control diagnoses	Methods	Stage of disease	Dysregulated miRNAs reported (n)	Country	Study
Serum	Laparoscopy for benign conditions	Dermoid cysts, serous cystadenoma, mucinous cystadenoma, simple ovarian cysts, paratubal cysts	Affymetrix miRNA Array & qRT-PCR	III/IV	10	USA, South Korea	Cosar et al. [17]
Serum	Laparoscopy for infertility	Infertility caused by tubal factors, paratubal cysts	Solexa sequencing & qRT-PCR	I/II	108	China	Wang et al. [69]
Serum	Laparoscopy for pain or infertility	Infertility caused by tubal factors; <i>note: excluded benign ovarian cyst</i>	Taqman miRNA Array & qRT-PCR	I/II/III/IV	6	China	Wang et al. [65]
Serum	Laparoscopy for benign conditions	Urinary incontinence, pelvic organ prolapse, ovarian haemorrhagic cyst	Microarray & qRT-PCR	II/III/IV	20	China	Hsu et al. [66]
Plasma	Laparoscopy for benign conditions	Uterine leiomyoma, mature teratoma, simple cysts, unexplained infertility	Agilent Human miRNA array & qRT-PCR	III/IV	6	China	Jia et al. [67]
Plasma	Two comparison groups: (1) endometriosis-associated ovarian cancer (2). Healthy, no symptoms	Pelvic pain with no signs of endometriosis	qPCR profiling (Human MiRNome Profiler kit) & qRT-PCR	Not given	23	USA	Surya-wanshi et al. [68]

[58, 59]. Extracellular microRNAs are either passively released or actively secreted from cells via exosomes or microvesicles, and may be complexed with lipoprotein or RNA-binding proteins such as Argonaute 2 [60, 61]. These lipid and protein complexes shield miRNAs from degradation by endogenous RNases. Circulating miRNAs have been compared to hormones in the context of cell–cell communication in tumor microenvironments [58], and it is logical that miRNAs could also impact endometriotic lesion development by mediating communication between eutopic endometrium and endometrial implants. Endometriotic lesions distantly located from the uterus can alter expression of genes in the eutopic endometrium [62], potentially mediated by circulating miRNAs. Recent studies have shown correlations between circulating miRNAs, such as those in the let-7 family, and the altered inflammatory signaling of endometriosis [63, 64].

To explore which circulating miRNAs have potential roles in endometriosis, global expression profiling of blood samples in endometriosis patients vs. distinct control groups has been undertaken, with six studies published to date (Table 1). These include three studies employing miRNA microarrays in patient serum samples [17, 65, 66], two using miRNA microarrays with plasma samples [67, 68] and one group that measured differential expression of miRNAs in serum with a deep sequencing approach [69]. In other recent work, investigators used qRT-PCR to quantify the expression of a specific set of circulating miRNAs in endometriotic serum [70–72] or plasma [73, 74]. These studies of circulating miRNAs in endometriosis are also summarized by Agrawal et al. and Panir et al. [53, 75].

In addition to analyzing miRNAs from different specimens (serum vs. plasma), many significant differences in study design

among the global expression studies complicate the ability to directly compare the results of these studies. These differences encompass the miRNA analysis method, subjects' genetic background, stage (I/II, minimal/mild vs. III/IV, moderate/severe) and type of endometriosis (ovarian, peritoneal, deep infiltrating), as well as the definition of the control groups (benign gynecological conditions, infertility, ovarian cancer or healthy controls). In addition, different research groups selected their cutoff points differently to define what they reported as a meaningful change in expression; a larger (i.e. 20-fold) expression change does not necessarily imply a more biologically significant effect than a smaller (e.g. 2-fold) change. Likewise, since miRNAs have many targets in cells, and a given mRNA transcript is subject to regulation by various miRNAs, the same pathway may be regulated by functionally redundant miRNAs, so lack of detection of a specific molecule does not preclude its involvement in the disease.

Despite these variables, the following circulating miRNAs were found to be dysregulated in two or more studies: members of the let-7 family (let7a-f), miR-9, miR-20a, miR-122, miR-141, miR-145, miR-199a, miR-342, miR-451a, and miR-3613 (Supplemental Table S1, [135]). The highest number of dysregulated miRNAs was reported in the Solexa sequencing study, with 108 candidate miRNAs, and there were substantial overlaps between these and the serum results reported from other groups [17, 65, 70]. Note that some groups reported supplementary lists of all dysregulated miRNAs detected, while others reported only the most altered sequences that were confirmed by qRT-PCR. This inclusion or exclusion affects the interpretation of overlap between studies.

Supplemental Table S1 lists 15 circulating miRNAs that were detected independently in more than one study of serum or plasma, and/or also displayed significant expression changes in both circulating and tissue studies, comparing either endometriotic lesions to eutopic endometrium or eutopic endometrium of endometriosis patients vs. controls. To be inclusive of the diverse published experiments related to miRNAs and endometriosis, Supplemental Table S1 includes one study reporting miRNAs dysregulated in endometrial mesenchymal stem cells (EN-MSCs) that were treated with sera of endometriosis patients [76] and two studies that compared fixed tissue samples from ovarian cancer tissue vs. endometrial and normal tissues [55] or cancer vs. paired endometriosis controls [77]. We also include a recent profile of miRNAs, cytokines, and other proteins in the peritoneal fluid from women with and without endometriosis, reporting 126 differentially expressed miRNAs identified by miRNA array analysis, with six of those validated by qRT-PCR [78].

While in some instances the reported direction of up- or downregulation of given miRNAs differs between studies, as discussed in [53], Supplemental Table S1 highlights several miRNA sequences that appear repeatedly as dysregulated in the disease, found by independent groups. The cross-validation of expression level changes across independent datasets demonstrates robust association in many instances. However, there is a significant degree of variability between the subjects and the design of these studies. To validate the significance of associations of dysregulated miRNAs with endometriosis, meta-analyses such as those conducted for genome-wide association studies in endometriosis could be performed. We acknowledge that certain studies may not be comparable and combining data across studies may introduce bias. When dealing with large-scale datasets, positive confounding may occur, which overestimates the association between variables. In addition, potential confounding variables such as demographics or clinical comorbidities should be accounted for when comparing results.

The miRNAs with altered expression in both blood and endometrial tissues are likely to be relevant to disease pathology and could prove to be suitable biomarkers for the disease. The functional roles of several of these repeatedly detected miRNAs have been examined in endometrial cell culture (Supplemental Table S2).

Functional studies on biological roles of miRNAs in endometriosis

Following large-scale miRNA expression studies that culminate in the identification of over- or underexpressed miRNAs in endometriosis patients, the specific roles of those miRNAs can be tested in hypothesis-driven functional studies. The effect of increased or decreased miRNA expression is tested in human cell lines or primary endometrial stromal cell (ESC) culture. Cultured cells are treated with inhibitors that knock down miRNA expression, or with exogenous miRNA to mimic overexpression, and then the expression levels (mRNA and protein) of predicted targets are measured. The targets of dysregulated miRNAs may be those predicted by bioinformatics tools [79] or inferred by previous work, and direct binding to the 3' UTR of these targets leading to translational inhibition may be tested using a luciferase assay [80]. Cellular phenotypes relevant to endometrial pathophysiology are often measured, such as cell proliferation, adhesion, migration, invasiveness, inflammatory signaling, or estrogen and progesterone signaling.

Supplemental Table S2 outlines functional investigations on individual miRNAs, carried out *in vitro*, which identified targets of 30

miRNAs related to endometriosis cellular processes. From the results of a PubMed database search using the terms “[miR or microRNA] and endometriosis” that retrieved 94 articles. This table lists articles, mostly published between 2012 and June 2018, in which endometriosis was the main focus (excluding studies that focused more on gynecological cancers). For further detail on studies conducted prior to 2012, the reader is encouraged to refer to the comprehensive reviews by Hull and Nisenblat on tissue and circulating miRNA in endometriosis [81] and by Ohlsson Teague et al. [82]. The most common pathway shown to be affected by downstream targets of miRNAs was cell proliferation (Supplemental Table S2). Other pathways impacted by miRNA dysregulation relate to apoptosis, angiogenesis, cell adhesion, invasion, or migration, inflammatory cytokine production, and steroid hormone signaling (estrogen signaling or progesterone resistance).

The most frequently identified mRNA target of endometriosis-related miRNAs was VEGFA, vascular endothelial growth factor A, found to be regulated by five miRNAs (Supplemental Table S2). This signaling protein stimulates angiogenesis, and is involved in blood vessel formation to endometriosis lesions. Regulation of angiogenesis by miRNAs, collectively referred to as angiomiRs, is well documented in tumorigenesis [83]. In endometriotic tissue expression studies investigating various angiogenesis-related miRNAs, the levels of VEGFA were also shown to differ based on the type of endometriotic lesion, with VEGFA protein detected at higher levels in peritoneal implants and rectovaginal nodules vs. endometriomas, suggesting more active angiogenesis than in ovarian endometriomas [42, 84]. VEGF receptor antagonists and other antiangiogenic agents have been explored as medical therapies for endometriosis [85]. In animal models of the disease, antiangiogenic drugs have been shown to suppress endometriotic tissue growth and decrease the number and volume of endometriosis lesions [85].

Matrix metalloproteinases (MMP-3 and MMP-9) are also repeatedly reported as targets of endometriosis-associated miRNAs, and the tissue inhibitor of metalloproteinase (TIMP) is also regulated by miRNAs (Supplemental Table S2). These proteolytic enzymes degrade extracellular matrix, contributing to tissue remodeling and invasion of endometrial cells into peritoneal tissue [86]. Altered expression of MMPs and TIMP has been documented in eutopic and ectopic endometrium, and MMP activity enhances the invasiveness of endometrial cells *in vitro* [87, 88]. Experiments profiling miRNA and mRNA expression from isolated eutopic ESCs also identified several of the same mRNA targets altered in women with endometriosis: ZEB1, MMP7, and TGF β 1 [89]. The latter is involved in the EMT, a well-known pathogenic mechanism in endometriosis that is regulated by several miRNAs [53]. In summary, processes such as cell proliferation, angiogenesis, extracellular matrix remodeling, inflammatory signaling, and hormonal regulation are documented in functional studies of miRNAs dysregulated in this disease.

MicroRNAs linked to endometriosis and infertility

The negative impact of endometriosis on fertility is well established; however, the molecular mechanisms responsible for these effects are still insufficiently characterized. Severe disease impacts fertility by direct physical interference; i.e. pelvic adhesions causing blockages between the ovary and fallopian tube, and further research is needed to elucidate how mild endometriosis leads to decreased endometrial receptivity and implantation [19]. Proposed mechanisms include abnormal folliculogenesis, immune and hormonal dysregulation, and increased oxidative stress [90]. MicroRNA studies have helped to

reveal details underlying the contribution of endometriosis to infertility.

Genes with altered expression in the eutopic endometrium of infertile women with endometriosis include HOXA10, aromatase, progesterone receptors (PGR), MMPs, and alphaV beta 3-integrin [91, 92]. As seen in Supplemental Table S2, miR-135a/b are upregulated during the proliferative phase in women with endometriosis, causing repression of the mRNA target, transcription factor HOXA10 [93]. As HOXA10 is required for endometrial receptivity [94], this is an early example showing how a dysregulated miRNA in endometriosis is linked to implantation failure [93]. The interplay between two noncoding RNAs, lncRNA H19 and let-7, was recently shown to influence the insulin-like growth factor (IGF) signaling pathway [91]. Long noncoding RNA H19 binds to let-7, and the decreased H19 seen in endometriosis increases let-7 activity in eutopic endometrium [91]. The authors postulate that this decreased IGF1 signaling reduces uterine stromal cell proliferation, leading to impaired endometrial receptivity [91].

Other miRNAs are implicated in the progesterone resistance that is characteristic of endometriosis (Supplemental Table S2). Progesterone resistance represents an integral link between endometriosis and fertility, as progesterone induces endometrial cell decidualization that is required for a normal pregnancy [19]. Progesterone resistance is associated with a failure to downregulate cell cycle regulators, increased cell proliferation, and a proinflammatory state in the endometrium [95]. In human endometriosis patients and in baboons with induced endometriosis, miR-29c expression levels were increased along with concurrent repression of progesterone receptor co-chaperone, FK506-binding protein 4 (FKBP4), and these expression changes were reversed after surgical excision of endometriosis [96]. Transfecting a miR-29c mimic into uterine fibroblast cells led to decreased decidualization markers and FKBP4 mRNA [96]. Our research has also shown that the diminished FKBP4 expression in endometrium of endometriosis patients is linked to impaired decidualization, via regulation by HOXA10 [97]. Another mechanism for progesterone resistance in endometriosis was uncovered by Zhou and colleagues, who reported upregulation of miR-196a and MEK/ERK signaling proteins in infertile women with minimal/mild endometriosis, and showed that this mediated downregulation of PGR expression [98]. In ESCs, this group demonstrated that adding an miR-196a mimic led to an increase in p-MEK/p-ERK proteins and a decrease in PGR protein, whereas the opposite trend was observed when an miR-196a inhibitor was used [98]. Recently, miR-194-3p was also demonstrated to downregulate PGR expression in the endometrium, contributing to progesterone resistance and reduced decidualization in women with minimal or mild endometriosis [99]. MiR-125b was shown to be regulated by progesterone, and through altering expression of one of its target genes, MMP26, can alter endometrial receptivity in women undergoing in vitro fertilization [100]. In conclusion, multiple miRNAs (such as let-7, miR-29c, miR-125b, miR-135a/b, miR-194, and miR-196a) are involved in pathways common to both endometriosis and infertility.

Circulating miRNAs with functional roles in endometriosis serve as potential biomarkers

Below we outline the functional evidence in detail for five miRNAs, identified as aberrantly expressed in multiple screens (Supplemental Table S1) and investigated in vitro for roles in endometriosis

(Supplemental Table S2). Three of these (let-7 family, miR-125b, and miR-451) were identified by our group as potential circulating biomarkers for the disease [70, 101], while other researchers have proposed miR-145 [74] and miR-199a as biomarkers [65, 102].

Let-7 family

Let-7 was the first human miRNA to be discovered [103]. The let-7 family of miRNAs consists of eight related sequences (let7a-i), which are known to be involved in early developmental events in humans and other animals. Let-7 regulates many basic processes of cell differentiation and can inhibit cellular reprogramming [104]. It has been classified as a tumor suppressor due to negative regulation of Ras family oncogenes, and the downregulation of the let-7 miRNA family is reported in many cancers [105]. The miRNA let-7b was detected to be downregulated in serum of endometriosis subjects by two research teams [65, 70]. In a murine model of endometriosis, circulating let-7a is also downregulated in serum [106]. In women with severe endometriosis, we previously reported a polymorphism in the let-7 binding site of the *KRAS* gene, enriched in women with severe endometriosis compared to disease-free controls [107]. As expected, loss of let-7 binding led to higher *KRAS* mRNA and protein levels, and decreased let-7 levels, in ESCs of women with this variant. *KRAS* activation has recently been observed in endometriosis patients and linked to endometriosis pathology and progesterone resistance [108]. Our research has also linked the let-7 family to the endometriosis therapeutic target, aromatase, involved in estrogen biosynthesis [109]. We showed that cells treated with an aromatase inhibitor had increased let-7 levels, while a let-7f mimic inhibited the expression of aromatase and reduced cell migration in vitro [109]. We recently reported that peritoneal infusion of let-7b reduced the size of endometriosis lesions [110]. Ongoing experiments aim to clarify the gene targets of the let-7 family miRNAs in endometriosis and test whether modulating these miRNAs may have potential therapeutic applications.

miR-125b

Another function of miRNAs in endometriosis involves altering inflammatory response. Both let-7b and miR-125b regulate inflammatory cytokine signaling, influencing levels of TNF-alpha, IL-6, and IL-1beta, which are increased in the circulation of women with endometriosis [63]. Elevated IL-1beta was shown in a prospective study to be associated with an increased risk of endometriosis [111]; IL-6 is highly expressed by endometriosis-associated macrophages, impacting cell migration in endometriosis development [112]. Along with the complementary roles of let-7 and miRNA-125 in inflammatory signaling, these miRNAs are also clustered together by their genomic location on the long arm of chromosome 21 (21q21.1) [113]. Let-7, miR-125b, and miR-200 are all involved in pathways of cancer progression, with miR-125b known to control apoptosis and cell proliferation [114]. Chang et al. found that miR-125b directly binds to BMPR1B, a tumor suppressor of ovarian cancer, and found single-nucleotide polymorphisms in this binding site that correlated with susceptibility to endometriosis [115]. Altered expression of miR-125b was also reported in ovarian endometriomas after leuprolide acetate (GnRH analog) treatment, suggesting its potential as a biomarker for monitoring treatment response [116].

miR-145

In ESCs and the endometriotic cell line 12Z, overexpression of miR-145 was shown to inhibit cell proliferation and invasiveness [117].

The expression of a number of target genes (pluripotency factors, cytoskeletal elements, and protease inhibitors) was altered in response to upregulated miR-145, and direct binding to JAM-A was demonstrated [117]. In ectopic endometrial tissue compared to eutopic endometrium within the same patient, Yang et al. reported higher miR-145-5p levels along with upregulation of VEGFA and decreased levels of EGFR2, PTEN, and CXCR4 [44]. Levels of miR-145 were found to be higher in women with stage I/II endometriosis vs. stage III/IV in one study [74], but levels did not differ between these disease stages in a separate study of infertile patients [118]. We detected increased serum miR-145 levels by microarray analysis; however, this expression difference between endometriosis patients and controls was not statistically significant in the follow-up qRT-PCR [17].

miR-199a

Similar to miR-145, overexpression of miR-199a reduced adhesion, migration, and invasiveness of ESCs [119]. The authors suggest a mechanism by which downregulation of this miRNA in endometriosis tissue attenuates cell invasiveness by suppressing the IKK/NF- κ B pathway and downregulating IL-8 [119]. EN-MSCs treated with overexpressed miR-199a-5p exhibited reduced VEGFA expression and decreased cell proliferation, motility, and angiogenesis [66]. This group also found that adding miR-199a-5p reduced the size of endometriotic lesions in a mouse model of the disease [66]. MiR-199a was found to increase in response to synthetic steroid hormones added to EN-MSCs from ovarian endometriomas, suggesting that this miRNA is involved in the mechanism of action of drugs like danazol that disrupt estrogenic hormone signaling and reduce lesion growth [120]. Further work on miR-199a explored the role of this miRNA under hypoxic conditions, in which it was shown to suppress VEGFA and hypoxia-inducible factor-1 α in ESCs and reduce angiogenesis [119]. In serum, one report found mi-199a expressed at higher levels in patients with severe endometriosis compared to mild disease [65], while another study showed no significant difference in this miRNA between rASRM stages [72].

miR-451

The role of miR-451 in endometriosis has been examined in several studies. It is upregulated in serum and tissues of both human patients and baboons with induced disease [17, 71, 121]. In an experimental mouse model, endometrial fragments from *miR-451a*-deficient mice were used to induce endometriosis and these *miR-451*^{-/-} cells formed a lower number of implants than normal endometrial fragments (*miR-451*^{+/+}), regardless of host genotype [122]. The *miR-451a*-deficient endometrial cells also expressed higher levels of fibrinogen alpha chain precursor, which contains three RGD cell adhesion motifs that can interfere with appropriate adhesion to the extracellular matrix, and may constrain the establishment of ectopic implants [122]. Another study confirmed the binding of miR-451a to the 3' UTR of macrophage inhibitory factor (MIF), which was downregulated in ectopic endometrial tissue compared to eutopic endometrium [123]. Overexpression of miR-451a in vitro led to decreased MIF and impaired cell survival, which the authors suggest may represent a mechanism to limit excessive cell proliferation in ectopic lesions [123]. In contrast, in the eutopic endometrium in a primate model of endometriosis, miR-451 expression was reduced [124]. Joshi and colleagues verified the ability of miR-451 to bind to YWHAZ, which codes for a 14.3.3 protein that suppresses apoptosis and enhances cell proliferation [124]. In a primate model of endometriosis, miR-451 levels were decreased in baboons treated

with simvastatin, suggesting the potential of this biomarker to be used to monitor response to endometriosis treatment [121].

Additional circulating miRNA biomarkers

In several studies including our own work, high sensitivity and specificity for diagnosing endometriosis have been demonstrated using receiver operating characteristic analysis. The area under the receiver operating characteristic curves, or AUC, can be used to define cutoff points for the level of each miRNA that can distinguish endometriosis from clinically relevant controls. While an AUC of up to 0.974 has been shown for single miRNAs in defined populations, using a panel of multiple miRNAs leads to improved sensitivity and specificity [65, 101]. Cosar et al. reported an AUC = 1.0 for a logistic regression model combining miR-125b-5p, miR-451a, and miR-3613-3p [101]. In a larger prospective study of women with all stages of endometriosis, our group observed that these and other previously identified biomarker candidates were differentially expressed in serum of women with endometriosis compared to controls with other benign gynecological diseases [125].

Wang et al. calculated an AUC of 0.994 for the combination of four miRNAs (miR-199a, miR-122, miR-145, and miR-542-3p) [65]. A recent prospective study reported that both miR-199a and miR-122 were upregulated in serum from women with endometriosis, showing high specificity for endometriosis [72], while a different group had reported significant downregulation of miR-199a in the serum of women with endometriosis [66]. The following other circulating miRNAs have been suggested as biomarkers in publications from 2018: miR-17 [126], miR-31 [74], and miR-154-5p [127]. A summary of the published AUC values for circulating miRNA biomarkers in endometriosis can be found in the 2018 review by Argawal et al. [53].

Conclusions

Recent advances in endometriosis research are beginning to show how miRNAs fit into the cellular pathways associated with the disease, strengthening the case for cell-free miRNA molecular signatures to serve as markers for detecting endometriosis. This review highlights a selection of miRNAs associated with the pathophysiology of endometriosis that are dysregulated both in endometrial tissues and in extracellular body fluids. Extracellular miRNAs meet many desirable criteria for biomarkers: they are stable in body fluids including blood and saliva; they display specific, distinct signatures compared to clinically relevant controls; there is also strong evidence of the biological relevance of circulating noncoding RNAs to this multifocal disease. Circulating biomarkers are also preferable to cellular biomarkers because samples can be collected from patients noninvasively.

Establishing the validity of these circulating biomarkers requires reproducible results from large patient populations, and wherever possible, an understanding of how their expression relates to disease progression. For biomarkers to be clinically valuable, they must provide novel, relevant information about the disease, or provide the same degree of information with less cost (monetary or risk-based) to the patient [128]. Several recent review articles have addressed the current challenges in pursuit of noninvasive diagnostic markers for endometriosis (see [129, 130] and [131]). Ideal diagnostic biomarkers would be present across the different subtypes of endometriosis (peritoneal, ovarian, and deep infiltrating), suggesting integral involvement in shared processes underpinning all clinical

manifestations of the condition. There are likely to be both universal markers common to all types of endometriosis, as well as a set of miRNAs that are more specific to each type [48]. Effective biomarkers should not be significantly impacted by sampling times of day or phase in the menstrual cycle, and should show consistent expression patterns in women from different ethnic backgrounds.

Conversely, there is also great potential to take advantage of the individual differences in expression patterns of noncoding RNAs, to help manage endometriosis from a personalized medicine standpoint. Specific miRNA readouts could assist clinicians in identifying the most effective endometriosis therapy for each individual and provide a way to track whether the disease recurs. The use of noncoding RNAs as diagnostic and prognostic biomarkers is promising in ovarian cancer [132] and colorectal cancer [133], as a strategy for patient stratification in clinical trials and predicting therapeutic response. When the first-line therapies, including NSAIDs and oral contraceptives, are not effective, a patient's unique miRNA expression pattern may help guide the decision of when to move on to second-line therapy (estrogen-suppressing drugs), or even to predict early on how effective a certain therapy may be. A high percentage of women fail to respond to progestin-based therapies and endometriosis has a high recurrence rate [134]. MicroRNA signatures could potentially help to identify these women earlier and steer them toward another treatment modality.

The changes in levels of circulating miRNAs in response to therapy were demonstrated in a recent study of the investigational drug simvastatin in a primate model of endometriosis [121]. Serum levels of three miRNA biomarkers (miR-150, miR-451a, and miR-3613) reverted to normal levels in baboons that underwent treatment [121]. Kiba and colleagues identified five miRNAs (miR-146a, miR-142-3p, miR-136, miR-125b-1, and miR-15b) that were upregulated in ovarian endometrioma tissue following treatment with leuprolide acetate (a GnRH analog) [116]. Therapies with different mechanisms of action will likely impact a different set of miRNAs, several of which have been identified in cell culture experiments. Treating cells with the aromatase inhibitor letrozole led to increased expression of let-7 family miRNAs [109]. Adding the steroid hormones danazol, progesterone, and medroxyprogesterone acetate (MPA) to EN-MSCs upregulated miR-34-5p and miR-199a-5p [120].

Longitudinal clinical studies, tracking biomarker expression in individual patients over time, will further clarify which miRNAs best correspond with the extent of disease and clearly correlate with therapeutic response and/or recurrence. As novel therapeutic approaches are tested, reliable biomarkers for active endometriosis could objectively measure how effectively the treatment reduces disease burden. Those that can be measured noninvasively, such as miRNAs, may prove to be instrumental to tracking how a patient responds to a particular drug. Future research may lead to miRNA-based tools for predicting treatment failure, as well as early identification of nonresponders, to initiate optimal treatment more quickly.

Combining expression analysis, in vitro studies, bioinformatic target prediction, and pathway mapping analysis with clinical findings will further solidify the profile of miRNAs involved in endometriosis. Several strong biomarker candidates have already been identified, which when used in combination could provide a compelling new tool for clinical use and help reduce the long diagnostic delay. Well-designed clinical validation studies in large, diverse populations will further confirm the potential of miRNA biomarkers to shift the paradigm of endometriosis diagnosis and management. Endometriosis clinical diagnosis is often delayed for many years after

symptom onset, leading to prolonged periods of untreated pain and disease progression. Given the wealth of information currently available and the validation of several miRNAs as markers of the disease, clinical implementation of a miRNA-based endometriosis diagnostic is urgently needed.

Supplementary data

Supplementary data are available at [BIOLRE](https://doi.org/10.1016/j.biolre.2019.05.001) online.

Supplemental Table S1. Common dysregulated miRNAs in circulating miRNA & tissue miRNA studies.

Supplemental Table S2. Functional studies of individual miRNAs and genes/pathways targeted.

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