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Non-coding RNAs in endometriosis: a narrative review

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BACKGROUND: Endometriosis is a benign gynaecological disorder, which affects 10% of reproductive-aged women and is characterized by endometrial cells from the lining of the uterus being found outside the uterine cavity. However, the pathophysiological mechanisms causing the development of this heterogeneous disease remain enigmatic, and a lack of effective biomarkers necessitates surgical intervention for diagnosis. There is international recognition that accurate non-invasive diagnostic tests and more effective therapies are urgently needed. Non-coding RNA (ncRNA) molecules, which are important regulators of cellular function, have been implicated in many chronic conditions. In endometriosis, transcriptome profiling of tissue samples and functional *in vivo* and *in vitro* studies demonstrate that ncRNAs are key contributors to the disease process.

OBJECTIVE AND RATIONALE: In this review, we outline the biogenesis of various ncRNAs relevant to endometriosis and then summarize the evidence indicating their roles in regulatory pathways that govern disease establishment and progression.

SEARCH METHODS: Articles from 2000 to 2016 were selected for relevance, validity and quality, from results obtained in PubMed, MEDLINE and Google Scholar using the following search terms: ncRNA and reproduction; ncRNA and endometriosis; miRNA and endometriosis; lncRNA and endometriosis; siRNA and endometriosis; endometriosis; endometrial; cervical; ovary; uterus; reproductive tract. All articles were independently screened for eligibility by the authors.

OUTCOMES: This review integrates extensive information from all relevant published studies focusing on microRNAs, long ncRNAs and short inhibitory RNAs in endometriosis. We outline the biological function and synthesis of microRNAs, long ncRNAs and short inhibitory

RNAs and provide detailed findings from human research as well as functional studies carried out both *in vitro* and *in vivo*, including animal models. Although variability in findings between individual studies exists, collectively, the extant literature justifies the conclusion that dysregulated ncRNAs are a significant element of the endometriosis condition.

WIDER IMPLICATIONS: There is a compelling case that microRNAs, long non-coding RNAs and short inhibitory RNAs have the potential to influence endometriosis development and persistence through modulating inflammation, proliferation, angiogenesis and tissue remodelling. Rapid advances in ncRNA biomarker discovery and therapeutics relevant to endometriosis are emerging. Unravelling the significance of ncRNAs in endometriosis will pave the way for new diagnostic tests and identify new therapeutic targets and treatment approaches that have the potential to improve clinical options for women with this disabling condition.

Key words: endometriosis / reproductive disorders / non-coding RNA / microRNA / short interfering RNA / long non-coding RNA / biomarkers / inflammation / ectopic endometrial tissue / endometrium

Introduction

Endometriosis is a benign gynaecological condition affecting ~10% of reproductive-aged women. Endometrial tissue implants and persists at ectopic locations, commonly presenting as lesions in the pelvic cavity (Giudice and Kao, 2004). Symptoms of painful periods, chronic pelvic pain and infertility occur, but since these are not specific for the presence of endometriotic lesions or disease severity, they are insufficient for a definitive diagnosis (Giudice, 2010; Parazzini *et al.*, 2012; Dunselman *et al.*, 2014). Due to a lack of informative biomarkers, the often early age of onset of symptoms and the symptomatic overlap with other conditions, diagnosis is commonly delayed. It has been estimated that definitive visual identification of lesions during surgery occurs between 5 and 10 years following the onset of symptoms (Hadfield *et al.*, 1996; Sinaii *et al.*, 2002; Greene *et al.*, 2009; Soliman *et al.*, 2017). Surgical removal of lesions can improve pain, but recurrence of symptoms occurs in 50% of women within 5 years of surgery (Guo, 2009). Suppression of menstruation is the most common medical intervention (Vercellini *et al.*, 2014; Brown and Farquhar, 2015) by means of combined oral contraceptive pills, progestins and GnRH agonists, often in conjunction with pain modifying agents. There is international recognition that accurate non-invasive diagnostic tests and more effective disease modifying agents are needed for endometriosis (Rogers *et al.*, 2016).

Sampson's theory that endometriosis arises when endometrial tissue in retrograde menstrual fluid implants in the pelvis, still prevails (Sampson, 1927). For a lesion to develop, interaction between cells of the endometrial tissue, the peritoneum and the immune system is required. Longitudinal characterizations of endometriosis-like lesions reveal a transition from acute inflammation and tissue breakdown, in the presence of interferon-gamma (IFNG) and tumour necrosis factor (TNF) (Koninckx *et al.*, 2012; Iwabe and Harada, 2014), to a state of tissue remodelling and repair, with lesions displaying proliferation, angiogenesis, neurogenesis and fibrosis under the influence of transforming growth factor B1 (TGFB1) (Omwandho *et al.*, 2010; Hull *et al.*, 2012; Dela Cruz and Reis, 2015). There is increasing evidence that non-coding RNAs (ncRNAs) mediate aspects of the complex dialogue between cells in these dynamic cytokine environments during endometriotic lesion development (Hull and Print, 2012; Sun *et al.*, 2014). For this reason, the potential of ncRNAs to improve our ability to diagnose and treat endometriosis has been vigorously investigated in recent years (Sun *et al.*, 2014; Nothnick *et al.*, 2015; Nothnick, 2017).

ncRNAs are functional RNAs transcribed from DNA, but not translated into proteins (Andersen and Panning, 2003; Hannon *et al.*, 2006). Since the first microRNA (miRNA) was identified in 1993 (Lee *et al.*, 1993), molecular tools that detect and map non-coding RNA have been developed to identify new ncRNA species. The ENCODE project, designed to delineate all functional elements encoded in the human genome, revealed that there were only 20 687 protein coding regions, representing 2.94% of the human genome (Moraes and Goes, 2016). Currently, within the human genome, 8801 small ncRNAs (<30 nucleotides (nt)) and 9640 long ncRNAs (>200 nt) have been identified (Moraes and Goes, 2016). Functional studies have demonstrated important roles for ncRNA in translation (small nucleolar RNAs (snoRNA)), mRNA processing (small nuclear RNAs (snRNA)), transposon repression and maintenance of germ line stability (PIWI-interacting RNA (piRNA)), regulation of gene expression (miRNA and short inhibitory RNAs (siRNA)), and chromatin modification and silencing (long ncRNA (lncRNA)) (Andersen and Panning, 2003; Bartel, 2004; Hannon *et al.*, 2006; Suh and Blelloch, 2011; Backofen and Vogel, 2014).

Functional experiments provide evidence that ncRNAs are critical components of the endometriotic disease process (Teague *et al.*, 2010; Gilabert-Estelles *et al.*, 2012; Hull and Print, 2012; Hull and Nisenblat, 2013; Yang and Liu, 2014; Zhao *et al.*, 2014a,b; Nothnick *et al.*, 2015). Genome-wide association studies have identified 19 independent genomic regions that display genome-wide significance for endometriosis risk (Uno *et al.*, 2010; Painter *et al.*, 2011; Nyholt *et al.*, 2012; Rahmioglu *et al.*, 2014a,b; Fung *et al.*, 2015; Sapkota *et al.*, 2015, 2017), the majority of which are in non-coding introns and intergenic regions. Thus ncRNAs are implicated in the heritable risk associated with endometriosis.

This review will summarize the biology of ncRNA species and describe our current understanding of the roles of miRNAs and lncRNAs in the pathophysiology of endometriosis. We also describe the use of siRNA in gene silencing or RNA interference (RNAi) approaches in *in vitro* and *in vivo* endometriosis models, since this is relevant to defining therapeutic targets and may itself comprise a tractable therapeutic approach. We will finally comment on the future potential diagnostic and therapeutic benefits of this technology for women with endometriosis.

Methods

Articles were selected for relevance, validity and quality, from search results obtained from various electronic databases, including PubMed, Medline, Google Scholar and EMBASE using the following terms and combinations thereof: ncRNA and reproduction; ncRNA and endometriosis; miRNA and endometriosis; lncRNA and endometriosis; siRNA and endometriosis; endometriosis; endometrial; cervical; ovary; uterus; reproductive tract. Further electronic database searching revealed key articles on the diagnostic and therapeutic use of ncRNAs.

Search results

A systematic literature search was carried out as detailed in the PRISMA flow diagram (Fig. 1) (Liberati et al., 2009). A total of 422 records were identified though database searching, with 223 unique articles selected for further screening. A total of 53 relevant primary articles were selected and assessed by K.P. and M.L.H. and 170 manuscripts were excluded from the systematic literature review, as detailed in Fig. 1.

MicroRNAs

MicroRNAs (miRNAs) are a highly conserved family of 19–22 nt sequences that regulate post-transcriptional gene expression. In the

21st release of miRBase, 28 645 hairpin miRNA precursors representing 35 853 mature miRNAs have been identified in 223 organism species (Kozomara and Griffiths-Jones, 2014). Functional experiments have shown that miRNAs participate in complex regulatory pathways to control development and maintain homoeostasis (Bartel, 2009).

miRNA exist as distinct transcriptional units or as clusters of polycistronic units able to generate multiple miRNAs (Bartel, 2004; Fazi and Nervi, 2008). miRNAs are transcribed in the nucleus as primary miRNA, where they undergo maturation steps that utilize the endonucleases, Drosha and Dicer, to attain functional capacity. Mature miRNA are then transported to their site of action following incorporation into the RNA-induced silencing complex (RISC) usually resulting in degradation of target mRNA or inhibition of translation (reviewed in greater detail in Bartel, 2004; Fazi and Nervi, 2008). There are also reports of miRNA acting to enhance target mRNA expression (Bueno and Malumbres, 2011; Green et al., 2016). miRNA actions are not limited to the local cellular environment. Several studies demonstrate that miRNAs can be transported into the systemic circulation in exosomes or microvesicles (Haider et al., 2014), where they can be incorporated into distant cells with functional consequences relevant to disease treatment (Bueno and Malumbres, 2011; Boon and Vickers, 2013). In addition, extracellular miRNAs associated with Argonaute proteins can be shielded from RNAse degradation, and are present at high concentrations in both blood plasma/serum and in tissue culture media (Arroyo et al., 2011;
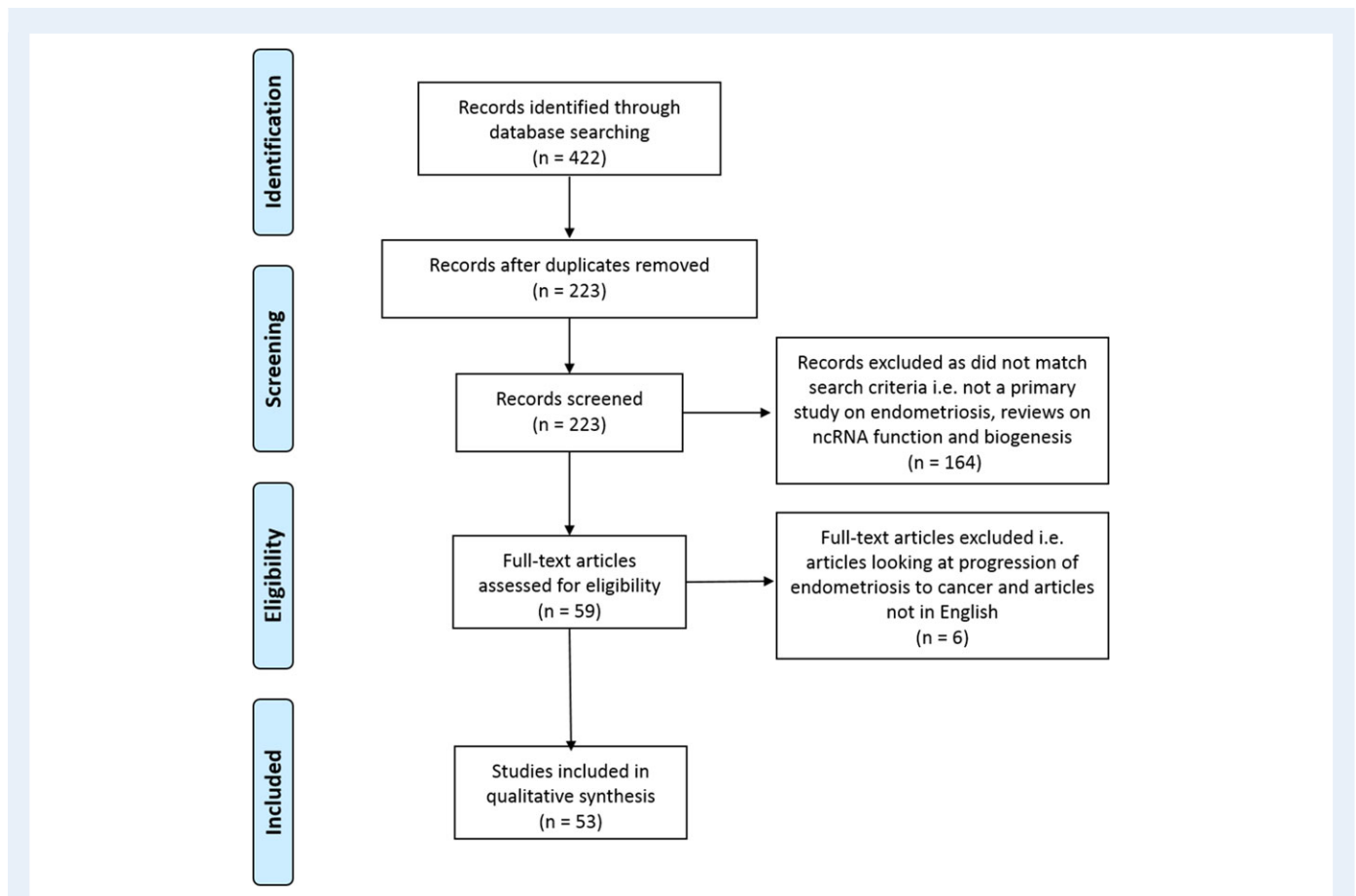

Figure 1 PRISMA flow methodology for selection of relevant manuscripts.

Turchinovich *et al.*, 2011; Meister, 2013). The high stability of miRNAs in circulation and distinctive changes in their plasma profile depending on various disease conditions strongly suggests that they may be ideal biomarkers of disease (Hayes *et al.*, 2014).

miRNAs in endometriosis

Multiple studies have shown that miRNA expression is altered in eutopic endometrium (Toloubeydokhti *et al.*, 2008; Burney *et al.*, 2009; Ramon *et al.*, 2011), in both ectopic and eutopic endometrial tissues (Toloubeydokhti *et al.*, 2008; Ohlsson Teague *et al.*, 2009; Filigheddu *et al.*, 2010) and in circulating miRNAs in women with endometriosis compared to healthy women (Hull and Nisenblat, 2013; Jia *et al.*, 2013; Wang *et al.*, 2013; Cho *et al.*, 2015; Rekker *et al.*, 2015). Furthermore, a range of functional studies, including the induction and modulation of miRNA expression levels and the use of luciferase assays *in vitro* (Hawkins *et al.*, 2011; Petracco *et al.*, 2011; Ramon *et al.*, 2011; Lin *et al.*, 2012; Abe *et al.*, 2013), suggest that discrete miRNAs may be able to regulate the dialogue between cellular components within endometriotic lesions, thereby contributing to their persistence. There are several caveats for the design of functional miRNA studies in investigating endometriosis. These include the facts that *in vitro* experiments must be performed in cell lines that express the specified microRNA, that singleplex PCR estimations can be unreliable as they lack a standardized control for miRNA and that single cell cultures do not reflect the complex cellular interplay seen in ectopic tissues. Informative *in vivo* experiments require novel mouse strains or specialized miRNA delivery methodologies that are able to modulate miRNA expression levels. However, as rodents do not menstruate, with the exception of the Spiny Mouse (Bellofiore *et al.*, 2017), these models are limited in that they do not spontaneously develop endometriosis and the disease has to be induced via transplantation of homologous uterine fragments. Efforts to circumvent the potential drawback of transplanting the myometrial tissue layer, which could affect disease progression, has resulted in the development of the 'menstrual' mouse model of endometriosis (Greaves *et al.*, 2014). However, in this model, as rodents are ovariectomised, the contribution of naturally cycling hormonal fluctuations on miRNA levels and lesion development cannot be effectively evaluated. Therefore, since no single model is entirely able to encapsulate the complexity of the human disease, a combination of functional studies *in vitro* and targeted evaluation of these miRNA *in vivo* should be carried out in tandem to help comprehend the mechanisms behind the pathogenesis of endometriosis and to exploit the potential of miRNAs as biomarkers of disease progression.

Eutopic endometrial tissue

A number of studies suggest that miRNAs are altered in eutopic endometrial tissue from women with endometriosis (Table I) (Burney *et al.*, 2009; Aghajanova and Giudice, 2011; Ramon *et al.*, 2011; Ruan *et al.*, 2013; Braza-Boils *et al.*, 2014; Zheng *et al.*, 2014). Initially, six downregulated miRNAs from the miR-9 and miR-34 families were identified when eutopic endometrium from women with endometriosis (*n* = 4) and without endometriosis (*n* = 3) (Burney *et al.*, 2009) was compared. Based on an *in silico* miRNA target analyses, miR-34 is thought to potentially regulate progesterone resistance and enhance proliferation and ectopic survival (Burney *et al.*, 2009).

Interestingly, miR-9 overexpression promotes breast cancer development (Gwak *et al.*, 2014), increases cell migration and invasiveness in SW480 human colon adenocarcinoma cells (Park *et al.*, 2016), and works in tandem with miR-124 to facilitate the conversion of human fibroblasts into neurons (Yoo *et al.*, 2011), suggesting that miR-9 may have an important role in the persistence of endometriosis lesions and associated nociception. A larger study, contrasting implantation phase eutopic endometrium from women with (*n* = 36) and without (*n* = 44) endometriosis, found upregulation of miR-29c, miR-200a and miR-145 in endometriosis patients (Ruan *et al.*, 2013). These miRNAs were postulated to contribute to implantation defects and endometriosis-associated subfertility. Additionally, comparison of mRNAs and miRNA profiles in eutopic endometrium from women with mild (*n* = 19) and severe (*n* = 44) endometriosis (Aghajanova and Giudice, 2011) has demonstrated upregulation of miR-21 throughout the menstrual cycle in patients with severe endometriosis, suggesting its use as a potential biomarker for disease progression. There is little overlap in the differentially expressed miRNAs identified by each of these studies, indicating a need for larger well-powered studies that adequately account for variation in clinical status and tissue biopsy composition to identify the candidate miRNAs that are most relevant for ongoing investigation. Given the lack of consensus amongst these studies, it is possible that further comparisons of eutopic tissue from women with and without endometriosis may demonstrate no substantial difference in miRNA expression patterns attributable to endometriosis. Furthermore, if differential expression of miRNAs in eutopic tissue was to be confirmed, it would be difficult to determine if it was an underlying causal factor driving initiation of disease or a consequence of altered eutopic tissue function that occurs secondary to lesion establishment.

Eutopic vs ectopic endometrial tissue

Many research groups have used microarrays or next-generation sequencing techniques to compare miRNA transcripts uniquely expressed within ectopic lesions (ovarian, peritoneal and/or rectovaginal) with paired or unpaired eutopic tissues from women with endometriosis or healthy controls (Table II) (Ohlsson Teague *et al.*, 2009; Filigheddu *et al.*, 2010; Hawkins *et al.*, 2011; Ramon *et al.*, 2011; Laudanski *et al.*, 2013; Braza-Boils *et al.*, 2014; Zheng *et al.*, 2014). The miRNAs identified again show limited concordance between experiments, which is likely to reflect the considerable heterogeneity in patient selection, experimental design, normalization methods and bioinformatic assessment of the studies. Additionally there is ongoing debate as to whether lesions at different locations represent different manifestations of the same disease process or distinct disease identities and heterogeneity between lesions from different locations could confound the molecular analyses (Borghese *et al.*, 2015). Across these studies, a total of 132 differentially expressed miRNAs were identified, with 23% of dysregulated miRNAs (31 miRNAs) being present in at least two of the studies (Ohlsson Teague *et al.*, 2009; Filigheddu *et al.*, 2010; Hawkins *et al.*, 2011; Ramon *et al.*, 2011; Laudanski *et al.*, 2013; Braza-Boils *et al.*, 2014; Zheng *et al.*, 2014). Collectively these data suggest that distinct miRNA profiles do indeed exist between ectopic and eutopic tissue, with predicted targets of these miRNA having multi-faceted roles in tissue remodelling, cellular proliferation and angiogenesis (Wei *et al.*, 2015).

Table I Studies evaluating altered miRNA expression in eutopic endometrial tissue from women with and without endometriosis.

Study	Sample description	Patient demographics					Dysregulated miRNAs
		Age	Ethnicity	Location of endometriosis	Grade	Cycle phase	
Burney et al. (2009)	Eutopic endometriosis (n = 4) vs eutopic controls (n = 3)	22–39 (mean 28)	Asian (n = 2), Caucasian (n = 1), Unknown (n = 1)	Peritoneal, rectovaginal, and ovarian (n = 4)	AFS III–IV	Early secretory	Downregulation of miR-9, 9*, 36b*, 34c-5p, 34c-3p and miRPlus_42 780
Aghajanova and Giudice (2011)	Eutopic severe endometriosis (n = 44) vs Eutopic mild endometriosis (n = 19)	20–48 (mean 35)	Caucasian (n = 42), African American (n = 2), Mixed/unknown (n = 8), Asian (n = 9), Asian Indian (n = 1), Hispanic (n = 1)	Unspecified (n = 63)	AFS I–IV	Mild endometriosis: Proliferative (n = 10), Early secretory (n = 3), Mid-secretory (n = 6); Severe endometriosis: Proliferative (n = 15), Early secretory (n = 12), Mid-secretory (n = 17)	Upregulation of miR-21 in severe vs mild endometriosis throughout the menstrual cycle; upregulation of DICER1 [involved in the biogenesis of miRNA] in the early and mid-secretory phases
Ramon et al. (2011)	Eutopic endometriosis (n = 41) vs eutopic controls (n = 38)	24–47 (mean 35)	Unspecified	Ovarian (n = 41); peritoneal (n = 24), rectovaginal (n = 13)	Unspecified	Proliferative (n = 26), Secretory (n = 32)	miR-15b, 16, 17-5p, 20a, 21, 125a, 221 and 222
Ruan et al. (2013) *Paper in Chinese	Eutopic endometriosis (n = 36) vs eutopic controls (n = 44)	N/A	N/A	N/A	AFS I–II (n = 17), III–IV (n = 19)	N/A	Upregulation of miR-29c, 145, 200a
Braza-Boils et al. (2014)	Eutopic endometriosis (n = 51) vs eutopic controls (n = 32)	20–45 (mean 34)	Caucasian	Ovarian (n = 51); peritoneal (n = 18), rectovaginal (n = 20)	Unspecified	Proliferative (n = 26), Secretory (n = 25)	Downregulation of miR-202-3p, 424-5p, 449-3p, 556-3p
Zheng et al. (2014)	Eutopic endometriosis (n = 22) vs eutopic controls (n = 22)	25–51 (mean 42)	N/A	Unspecified (n = 22)	Unspecified	Proliferative (n = 10), Secretory (n = 12)	Upregulation of miR-143, and 145

Table II Studies evaluating altered miRNA expression in eutopic vs ectopic endometrial tissue.

Study	Sample description	Patient demographics				Dysregulated miRNAs
		Age	**Location of endometriosis**	**Grade**	**Cycle phase**	
Ohlsson Teague et al. (2009)	Eutopic vs ectopic in endometriosis (n = 7)	Unspecified	Peritoneal (n = 7)	AFS Stage II–IV	Proliferative (n = 4), Secretory (n = 3)	Upregulated: miR-145, 143, 99a, 99b, 126, 100, 125b, 150, 125a, 223, 194, 365, 29c and 1 Downregulated: miR-200a, 141, 200b, 142-3p, 424, 34c, 20a and 196b
Filigheddu et al. (2010)	Eutopic vs ectopic in endometriosis (n = 3)	24–48 (average 36)	Ovarian (n = 3)	ASRM III–IV	Early proliferative	Upregulated: miR-1, 100, 101, 126, 130a, 143, 145, 148a, 150, 186, 199a, 202, 221, 28, 299-5p, 29b, 29c, 30e-3p, 30e-5p, 34a, 365, 368, 376a, 379, 411, 493-5p and 99a Downregulated: miR-106a, 106b, 130b, 132, 17-5p, 182, 183, 196b, 200a, 200b, 200c, 20a, 25, 375, 425-5p, 486, 503, 638, 663, 671, 768-3p, 768-5p and 93
Hawkins et al. (2011)	Ectopic endometriosis (n = 10) vs eutopic controls (n = 11)	20–48 (median 34)	Ovarian (n = 10)	Unspecified	Proliferative (n = 6), secretory (n = 1), interval phase (n = 1), prior hysterectomy (n = 2)	Upregulated: miR-202, 193a, 29c, 708, 509-3-5p, 574-3p, 193a-5p, 485-3p, 100 and 720 Downregulated: miR-504, 141, 429, 203, 10a, 200b, 873, 200c, 200a, 449b, 375 and 34c-5p
Ramon et al. (2011)	Paired eutopic vs ectopic in endometriosis (n = 41); ectopic endometriosis (n = 41) vs eutopic controls (n = 38)	24–47 (mean 35)	Ovarian (n = 41); peritoneal (n = 24), rectovaginal (n = 13)	Unspecified	Proliferative (n = 26), secretory (n = 32)	miR-15b, 16, 17-5p, 20a, 21, 125a, 221 and 222
Laudanski et al. (2013)	Ectopic endometriosis (n = 21) vs eutopic controls (n = 25)	20–35	Ovarian (n = 21)	AFS III–IV	Proliferative	Upregulated: miR-24 and 885-5p Downregulated: miR-26b, let-7b, 185, 142-3p, 29b, 483-5p, 144*, 145*, 629*, 222*, 497, 675 and 106b*
Braza-Boils et al. (2014)	Paired eutopic vs ectopic in endometriosis (n = 51); Ectopic endometriosis (n = 51) vs eutopic controls (n = 32)	20–45 (mean 34)	Ovarian (n = 51); peritoneal (n = 18), rectovaginal (n = 20)	Unspecified	Proliferative (n = 26), secretory (n = 25)	miR-16-5p, 29c-3p, 138-5p, 202-3p, 373-3p, 411-5p, 411-3p, 424-5p, 449b-3p, 556-3p, 636, 935
Zheng et al. (2014)	Paired eutopic vs ectopic in endometriosis (n = 11); ectopic endometriosis (n = 22) vs eutopic controls (n = 22)	25–51 (mean 42)	Unspecified (n = 22)	Unspecified	Proliferative (n = 10), secretory (n = 12)	Upregulated: miR-143 and 145

Table III Studies evaluating altered miRNA expression in blood samples from women with and without endometriosis.

Study	Sample description	Patient demographics					Dysregulated miRNAs
		Age	Ethnicity	Location of endometriosis	Grade	Cycle phase	
Jia et al. (2013)	Plasma miRNA microarray from women with endometriosis (n = 3) vs without (n = 3). Validation in 20 patients and 20 controls.	25–44 (mean 34)	Unspecified	Unspecified (n = 23)	ARSM III (n = 10), IV (n = 13)	Proliferative (n = 19), secretory (n = 4)	Downregulated: miR-122, 17-5p, 19, 20a, 19b, 15b-5p, 451, 30e, 27a, 92, 26a, 320e, 320d, 762, 1274b, 630, 29c, 223, 22, 1268, 23a, H10, 21, 3679-5p, 30d, 572 and 3665.
Suryawanshi et al. (2013)	Plasma miRNA microarray from women with endometriosis (n = 7) vs without (n = 6). Validation in 33 patients and 20 controls.	mean 36	White (n = 30), unspecified (n = 3)	Unspecified (n = 33)	Unspecified	Unspecified	Upregulated: miR-16, 195, 191, 1974, 4284, 15b, 1978, 1979, 362-5p and 1973.
Wang et al. (2013)	Serum miRNA microarray from women with endometriosis (n = 10) vs without (n = 10). Validation in 60 patients and 25 controls.	20–58 (mean 34)	Unspecified	Ovarian (n = 41), peritoneal (n = 19)	AFS I (n = 17), II (n = 5), III (n = 14), IV (n = 24)	Proliferative (n = 42), Secretory (n = 18)	Upregulated: miR-377-5p, 378, 193a-3p, 504, 708, 122, 130a, 199a, 330-5p, 218-1*, 1180, 520d-3p, 215, 369-3p, 561, 203, 365, 1275, 377*, 302a, 647, 455, 367, 651 and 122* Downregulated: miR-1825, 139-3p, 141*, 489, 1243, 145*, 9*, 128a, 548b-5p, 572, 758, 202, 20a*, 654-3p, 18a*, 19b-1*, 185, 511, 335*, 515-3p, 548c-5p, 27a*, 645, 324-3p, 204, 886-5p, 384, 296, 323-3p, 598, 10b*, 206, 520c-3p, 1291, 542-3p and let-7b.
Hsu et al. (2014)	Serum from women with vs without endometriosis (40 patients, 25 controls)	18–53 (mean 35)	Unspecified (n = 40)	Ovarian (n = 36), peritoneal (n = 4)	ASRM II (n = 5), III (n = 9), IV (n = 26)	Unspecified	Upregulated: miR-18a, 518e, 92a, 181d, 516a-3p, 423-3p, 212, 219-1-3p, 187 and 548-5p Downregulated: miR-199a-5p, 490-5p, 488, 302d, 139-3p, 518c, 320a, 425, 214 and 151-3p
Cho et al. (2015)	Serum miRNA expression from women with endometriosis (n = 24) vs without (n = 24).	18–48 (mean 33)	Unspecified	Ovarian (n = 24 [100%]), peritoneal (n = 22 [91.6%]); deep infiltrating endometriosis (n = 8 [33.3%])	ASRM III (n = 11), IV (n = 13)	Proliferative and secretory	Downregulated: miR-135a, let-7b, let-7d and let-7f
Rekker et al. (2015)	Plasma miRNA expression from women with endometriosis (n = 61) vs without endometriosis (n = 65) at either morning or evening blood collection times.	mean 33	Unspecified (n = 61)	Ovarian and/or peritoneal	ASRM I–II (n = 33); III–IV (n = 28)	Unspecified	Downregulated: miR-200a-3p and miR-141

Continued

Table III Continued

Study	Sample description	Patient demographics		Location of endometriosis	Grade	Cycle phase	Dysregulated miRNAs
		Age	Ethnicity				
Cosar et al. (2016)	Serum from women with endometriosis (n = 24) vs without endometriosis (n = 24).	20–50	Unspecified	Ovarian and/or peritoneal	ASRM III–IV	Proliferative (n = 8) and Secretory (n = 16)	Upregulated: miR-125-5p, 150-5p, 342-3p, 145-5p, 143-3p, 500a-3p, 451a and 18a-5p, Downregulated: miR-6755-3p and 3613-5p
Wang et al. (2016a,b)	Serum from women with endometriosis (n = 30) vs without endometriosis (n = 20).	22–48 (mean 33)	Unspecified	Ovarian and/or peritoneal	AFS I–II	Proliferative (n = 19) and Secretory (n = 11)	Upregulated: miR-424-3p, 550a-3p, 296-5p, 502-3p, 542-3p, 185-5p, 3127-5p, 24-2-5p, 636 and 4645-3p Downregulated: miR-889, 432-5p, 381, 410, 584-5p, 99b-5p, 127-3p, 30c-5p, 215 and let-7a-5p
Nothnick et al. (2017)	Serum from women with endometriosis (n = 41) vs without endometriosis (n = 40).	23–44	Unspecified	Ovarian and/or peritoneal	ASRM I/II (n = 12); III/IV (n = 29)	Proliferative (n = 16) and Secretory (n = 25)	Upregulated: miR-451a

Amongst the differentially expressed miRNAs, miR-29c, miR-100 and miR-143 have emerged as consistently upregulated in ectopic endometrial tissues in four studies (Ohlsson Teague et al., 2009; Filigheddu et al., 2010; Hawkins et al., 2011; Zheng et al., 2014). miR-29c, which is known to regulate genes controlling endometrial cell proliferation, apoptosis and invasion (Ohlsson Teague et al., 2009; Filigheddu et al., 2010; Hawkins et al., 2011), targets c-Jun during the late secretory phase (Udou et al., 2004; Long et al., 2015). This is postulated to upregulate anti-apoptotic mechanisms in stromal cells, thereby promoting cellular survival during disease establishment (Long et al., 2015). Upregulation of miR-100 has been found to inhibit cellular proliferation, migration and invasion in a cancer model, whereas downregulation promoted metastasis (Zhou et al., 2014a,b). Similarly, miR-143 is associated with the development of endometrioid carcinomas (Wang et al., 2014). The upregulation of miR-100 and miR-143 in endometriotic tissues is hypothesized to confer protection from malignant change and promotion of a benign phenotype of endometriosis.

Circulating miRNAs

Although endometriosis is a common gynaecological disorder, establishing a reliable non-invasive diagnostic test remains challenging. The potential for utilizing miRNAs as serum diagnostic markers of disease progression has prompted analysis of dysregulated miRNAs in blood of women with endometriosis. To date, seven studies have examined circulating miRNAs using high throughput assays in either serum (Wang et al., 2013, 2016a,b; Hsu et al., 2014; Cho et al., 2015; Cosar et al., 2016) or plasma samples (Jia et al., 2013; Suryawanshi et al., 2013) taken from women with and without endometriosis (Table III) (Jia et al., 2013; Suryawanshi et al., 2013; Wang et al., 2013, 2016a,b; Hsu et al., 2014; Cho et al., 2015; Rekker et al., 2015; Cosar et al., 2016). A further two papers have used singleplex RT-PCR assay methods to demonstrate downregulation of the mir-200 family in plasma (Rekker et al., 2015) and upregulation of miR-451a levels in women with endometriosis (Nothnick et al., 2017) (Table III). The results generally show little consistency between these studies. Although several studies identify circulating miRNAs with sensitivities and specificities high enough to suggest utility as a diagnostic tool, the heterogeneity in experimental design, specimen collection, bioinformatic analysis and normalization methods make the findings difficult to replicate. None of the circulating miRNA tests have been successfully trialled in a large, independent test cohort of women with and without endometriosis.

Plasma levels of miR-200a and miR-141 were identified as potential biomarkers for endometriosis, but expression levels were found to be altered in response to the time of the day at which blood collection occurred (Rekker et al., 2015). It may be that the impact of circadian rhythms on plasma miRNA levels is a key factor accounting for inconsistency between studies. Menstrual cycle phase has also been raised as a potential confounding factor, but on investigation, no significant variation in plasma miRNAs across the menstrual cycle was found in one study (Rekker et al., 2015). Notwithstanding, it seems prudent that standardization of sampling practices and assays for assessment of plasma miRNAs in large cohorts is required to better progress development of informative diagnostic markers.

Endometriosis has the potential to progress to endometriosis-associated ovarian cancer (EAOC), and plasma miRNAs may prove
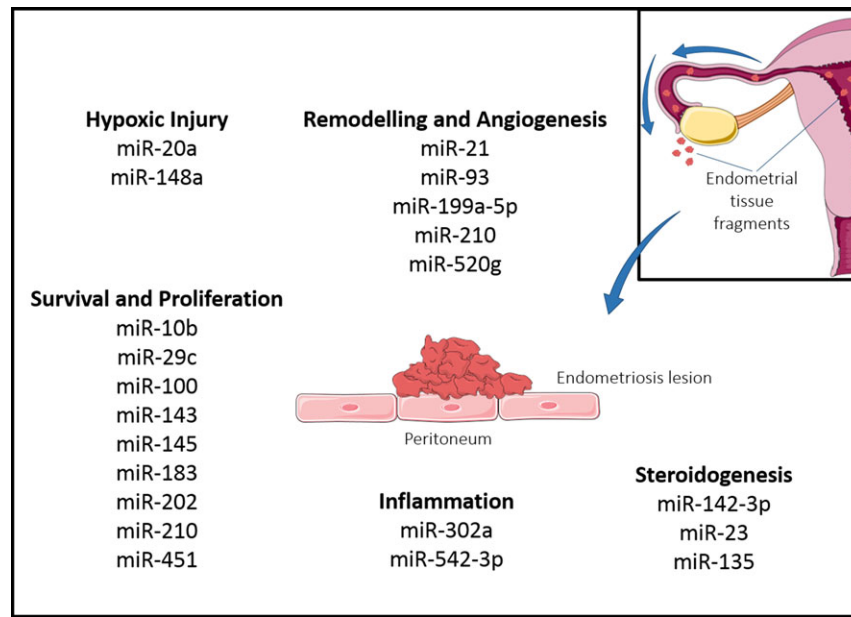
Figure 2 MicroRNAs implicated in the development of endometriosis. Experimental validation of microRNAs in endometriosis have shown that multiple biological process are regulated by miRNAs and may have significant impacts on lesion development.

to be markers for malignant disease progression (Li et al., 2010; Okada et al., 2010; Dinulescu, 2012; Viganò et al., 2012; Suryawanshi et al., 2013; Králíčková and Vetvicka, 2014; Yan et al., 2014; Zhao et al., 2014a,b). Suryawanshi et al. (2013) compared plasma miRNA levels from women with endometriosis to those with EAOC as well as healthy controls (Suryawanshi et al., 2013). Ten miRNAs were differentially expressed between the three groups, all being higher in patients with endometriosis.

Pathophysiological pathways impacted by differentially expressed miRNAs

The miRNAs identified as dysregulated in endometriosis appear to target mRNAs involved in a range of cellular and biological pathways, several of which are logically implicated in endometriotic lesion development (Fig. 2).

Hypoxic injury
Hypoxia characterizes the early phases of ectopic endometrial tissue survival and hypoxia induced factor 1-α (HIF-1α) gene expression is upregulated in endometriotic tissues (Chen et al., 2015) and in early stage endometriosis-like lesions from mouse models. In endometriotic lesions, high levels of miR-20a prolong HIF-1α activation of extracellular-signal-regulated kinase (ERK) (Lin et al., 2012), inducing a signalling cascade which increases fibroblast growth factor (FGF)-9 expression. FGF-9 stimulates endothelial and endometrial stromal cell proliferation and angiogenesis, potentially contributing to ectopic lesion development (Tsai et al., 2002). Elevated miR-20a expression suppresses antiangiogenic Netrin-4 gene expression (Zhao et al., 2014a,b), potentially enhancing angiogenesis in ectopic endometrial lesions. Hypoxic conditions in endometriotic lesions also induce miR-148a

and AU-rich element binding factor 1 (AUF1) expression in vitro (Hsiao et al., 2015), leading to destabilized DNA methyltransferase 1 mRNA expression. This could account for the aberrant epigenetic methylation patterns seen in endometriosis patients.

Inflammation
Aberrant immune surveillance is thought to reduce the clearance of endometrial issue within the peritoneal cavity permitting attachment, progression and subsequent disease persistence (Herington et al., 2011; Králíčková and Vetvicka, 2015). The inflammatory mediators interleukin-1β (IL-1B) (Milewski et al., 2008), TNF (Keenan et al., 1995; Gmyrek et al., 2008) and cyclooxygenase (COX)-2 (Wu et al., 2002) are elevated in peritoneal fluid and ectopic lesions from women with endometriosis and their inhibition suppresses endometriotic-like lesion development in animal models (Dogan et al., 2004; Kyama et al., 2008). Interestingly, there are studies that suggest that these inflammatory mediators can be targeted by miRNAs in endometrial tissue, which might then contribute to development of endometriosis. For example, Toloubeydokhti et al. (2008) discovered that miR-542-3p interacts with and downregulates COX-2 in ectopic endometrial tissues (Toloubeydokhti et al., 2008). Furthermore, IL-1B, COX-2 and TNF are indirectly targeted by miR-302a in endometrial stromal cells (ESCs), where miR-302a suppression of chicken ovalbumin upstream promoter-transcription factor II results in induction of these inflammatory mediators (Lin et al., 2014).

Steroidogenesis
Aberrant estrogen and progesterone biosynthesis, metabolism and sensitivity appear to contribute to the development of endometriosis (Bulun et al., 2012). For example, aromatase activity is upregulated in endometriotic lesions as part of a feed forward loop involving COX-2,

increasing local estrogen production and promoting endometriosis development. Increased miR-142-3p levels in primary ESCs reduced steroid sulfatase and IL-6 activity, suggesting a dual effect on steroidogenic and inflammatory pathways in endometriosis (Kastingschafer et al., 2015).

Overexpression of miR-23a and miR-23b which target the NR5A1 gene, leads to the repression of steroidogenic factor-1, resulting in reduced levels of aromatase P450 and steroidogenic acute regulatory protein (Shen et al., 2013). Expression of these miRNAs expression is reduced in eutopic and ectopic endometrium from women with endometriosis (Shen et al., 2013), which is predicted to enhance estrogen synthesis, promote proliferation in endometriotic tissues and delay endometrial transition from the proliferative to secretory phase, which manifests as progesterone resistance (Gilabert-Estelles et al., 2012; Shen et al., 2013). Progesterone resistance may also be promoted by other miRNA which are increased in eutopic endometrium of endometriosis patients. miR-135a and miR-135b for example, both target the HOXA10 gene and are involved in uterine stromal cell responsiveness to progesterone (Petracco et al., 2011).

Cell proliferation, survival and invasion
Mouse models demonstrate that cellular proliferation is important for the survival and growth of endometrial fragments at ectopic sites (Bruner-Tran et al., 2012; Khanjani et al., 2012; Winterhager, 2012) and miRNA regulation is important to this process. For instance, high expression of miR-210 in ESCs results in signal transducer and activator of transcription 3 (STAT3) activation and increased proliferation, angiogenesis and resistance to apoptosis (Okamoto et al., 2015), whereas upregulation of miR-202 modulates sex determining region Y-box 6 expression, increasing the proliferation and invasiveness of ESCs (Zhang et al., 2015). Suppression of miR-196b increases the proliferative capacity and anti-apoptotic mechanisms of endometriotic cells *in vitro* (Abe et al., 2013). Further, the invasive potential of ESCs is enhanced by miR-183 suppression, which increases integrin β1 expression, a vital component of cell adhesion (Shi et al., 2014; Chen et al., 2015). Similarly, miR-10b and miR-145 increase ESC proliferation and invasiveness by targeting multiple cytoskeletal elements and metalloproteinases (Adammek et al., 2013; Schneider et al., 2013).

In human endometriotic lesions, miR-451 expression was inversely correlated with the expression of macrophage migration inhibitory factor (MIF), which contributes to endometrial epithelial cell survival (Graham et al., 2015). Similarly, reduced expression of miR-451 in lesions from a baboon model of endometriosis (Joshi et al., 2010), corresponds to increases in expression of its predicted targets CDKN2D, GATAD2B and YWHAZ. A recent study also found a significant increase in miR-451a levels in serum from women with endometriosis, as well as in baboons with experimentally induced endometriosis (Nothnick et al., 2017). Tumour suppressor activity associated with miR-451, including regulation of NOTCH1-induced oncogenesis (Li et al., 2011) and the modulation of IKKβ (Li et al., 2013) and IL6R (Liu et al., 2014) gene expression, also likely contribute to the increased proliferation and anti-apoptotic responses seen in endometriotic lesions. This hypothesis was tested in the only *in vivo* functional miRNA study to date which has utilized a mouse model of endometriosis (Nothnick et al., 2014). Uterine fragments from miR-451-deficient mice were transplanted to induce endometriosis-like lesions in genetically normal recipients. Ectopic attachment and

survival of lesions appeared to be impaired with fewer lesions observed in miR-451 deficient implants, confirming that miR-451 confers protection from host clearance mechanisms (Nothnick et al., 2014).

Tissue repair, remodelling and angiogenesis
Several factors that promote tissue repair, remodelling and angiogenesis appear to be targeted by miRNAs in endometriosis. Vascular endothelial growth factor (VEGF) is a critical angiogenic factor expressed in endometriotic tissues and peritoneal macrophages (Laschke and Menger, 2007; Groothuis, 2012; Krikun, 2012) and its inhibition in animal models of endometriosis suppresses lesion development (Hull et al., 2003; Nap et al., 2005). miR-210 which is induced in ESC cultures, contributes to VEGF regulation as miR-210 transfection results in induction of VEGF-A and STAT3 (Okamoto et al., 2015), resulting in increased angiogenesis, cell proliferation and reduced apoptosis.

miR-21 and miR-199a-5p also appear to contribute to VEGF regulation in endometriosis. miR-21 is expressed at high levels in exosomes released from primary ESCs, and its overexpression was found to upregulate VEGF leading to enhanced angiogenesis (Harp et al., 2016). Upregulation of miR-199a-5p was shown to repress VEGF-A expression in endometrial mesenchymal stem cells, causing cell proliferation and angiogenesis to be inhibited (Hsu et al., 2014). Functional validation in a mouse model confirmed the pathophysiological relevance of this miRNA, with a reduction in endometriosis-like lesions following repeated delivery of pre-miR-199a (Hsu et al., 2014).

There is evidence that matrix metalloproteases (MMPs), which are elevated in endometriosis lesion, are also regulated by miRNAs (Groothuis, 2012). These include miR-93, the expression of which is suppressed and inversely correlated to MMP3 and VEGF-A bioactivity in eutopic endometrial cells from women with endometriosis (Lv et al., 2015). Furthermore, systematic evaluation of 17 single nucleotide polymorphisms (SNPs) in the *MMP2* gene identified an aberrant miR-520g binding site which is associated with endometriosis susceptibility (Tsai et al., 2013). It was postulated that this SNP predisposes to endometriosis by creating deficiency in the regulatory action of miR-520g on MMP2 synthesis. In this scenario, unregulated levels of MMP2 could act to enhance degradation of the extracellular matrix and facilitate anchoring of endometrial fragments to ectopic sites and subsequent tissue remodelling (Tsai et al., 2013).

Experimental validation of miRNAs in endometriosis

Better understanding of the specific targets and biological functions of differentially abundant miRNAs in eutopic and ectopic endometrial tissues and peripheral blood has potential to yield insight on the underlying pathophysiological mechanisms of endometriosis, and to identify therapeutic targets. However, there are challenges to unravelling the key targets of miRNAs, particularly since most miRNAs are highly pleiotropic in their identified targets. A large majority of postulated miRNA–mRNA interactions require additional experimental identification and/or verification, as identification of the mRNA targets of miRNAs, based solely on computational algorithms and sequence-based predictions can result in false positive hits. Thus, the real contribution of each of the miRNAs and their pathways of action described above will require investigation in animal models or by

other experimental approaches that allow the impact of perturbation of specific miRNAs on disease progression to be investigated.

Long non-coding RNAs

Most RNAs within the non-coding transcriptome (Hannon et al., 2006; Kung et al., 2013; Mattick and Rinn, 2015) are lncRNA, with more than 9000 identified. These ncRNAs localize to the nucleus or cytoplasm and can be divided into a number of subtypes, including (i) long intergenic ncRNAs (lincRNAs) transcribed by RNA polymerase II from intergenic regions of the genome, (ii) natural antisense transcripts (NATs) transcribed either as an entire or partial antisense transcript to coding genes, (iii) 3′ UTR-associated RNA (uaRNA) derived from cleavage of long precursors of mRNA transcripts and (iv) enhancer RNAs (eRNAs) derived from bidirectional transcription of enhancer domains (Mattick and Rinn, 2015).

lncRNAs form base pairs with DNA and RNA to control transcription and post-transcriptional processing (Kung et al., 2013) by masking or enhancing the function of splice junctions, miRNA-binding sites and promoter sites. These ncRNAs can also fold to form structural domains that interact with proteins (Kung et al., 2013; Mattick and Rinn, 2015) such as polycomb repressive complex (PRC2) which is recruited to chromatin to methylate histone proteins during epigenetic regulation (Kung et al., 2013; Taylor et al., 2015). lncRNA were initially thought to be only *cis* response elements, regulating the transcription of nearby genes, however, numerous trans-acting lncRNA have now been described (Zhang et al., 2013).

lncRNAs that modulate estrogen, androgen and progesterone receptor responses have been identified, suggesting a role for lncRNAs in attenuating hormone regulatory networks in endometrial function (Taylor et al., 2015). For example, lncRNA H19 expression is restricted to the ovaries and endometrium, where it displays menstrual cyclicity and proliferative phase upregulation (Ariel et al., 1997). The H19 gene is imprinted, being transcribed but not translated from the maternal allele (Brannan et al., 1990), whereas the paired, co-located gene for insulin-like growth factor 2 (IGF2) is only expressed paternally (Zemel et al., 1992). In implantation phase endometrial samples, H19 was 4-fold lower and IGFR2 mRNA levels slightly higher in women with unexplained infertility, implying a role in implantation (Gao et al., 2012; Keniry et al., 2012). lncRNAs are also described with functions in the TNF and TGFB1 signalling pathways, which are known to be critical for endometriosis lesion development (Murray et al., 2014; Iwabe and Harada, 2014; Zhou et al., 2014a,b; Králíčková and Vetvicka, 2015).

lncRNAs as biomarkers of endometriosis

The first study investigating lncRNA function in endometriosis demonstrated reduced levels of H19 lncRNA in the eutopic endometrium of women with endometriosis compared to healthy controls (Ghazal et al., 2015). By acting as a molecular sponge, it appears that H19 reduces the bioavailability of miRNA let-7 and its downstream target insulin-like growth factor 1 receptor (IGF1R), to reduce the proliferation of endometrial stroma cells (Ghazal et al., 2015). The H19/Let-7/IGF1R regulatory pathway is the first to link a dysregulation in lncRNA to miRNA activity in endometriosis, raising the prospect that interactions between different ncRNA subsets contribute to the endometriotic disease process.

Microarrays with lncRNA oligonucleotide probes have now identified several additional ncRNA as being dysregulated in endometriosis (Sun et al., 2014; Ghazal et al., 2015; Wang et al., 2015, 2016a,b) (Fig. 3). When four paired samples of eutopic endometriosis and endometrioma tissues were compared, 948 lncRNA and 4088 mRNA dysregulated transcripts were identified (Sun et al., 2014). Three of these lncRNAs were associated with cis regulatory HOXA mRNA clusters. Consistent with a role for these lncRNA impacting gene expression, HOXA10 mRNA was downregulated in ectopic endometrium of women with endometriosis, although this might also reflect aberrant methylation (Wu et al., 2005). Given HOXA10 was similarly downregulated in eutopic endometrium in endometriosis, this could impact both endometrial receptivity and the ability of endometrial tissue to implant ectopically. Thus it seems that HOXA10 regulators include lncRNAs in addition to hypermethylation and reproductive steroid hormones (Lim et al., 1999; Wu et al., 2005; Kulp et al., 2012).

In an additional study, eutopic endometrial samples taken in in the late secretory phase from three women with and without endometriosis were compared (Wang et al., 2015). The 1277 dysregulated lncRNAs (488 upregulated and 789 downregulated) were then subclassified as lincRNA ($n = 54$), NATs ($n = 13$) and eRNAs ($n = 25$) and combined in an analysis with 1216 differentially regulated mRNAs (578 upregulated and 638 downregulated), in a coding–noncoding gene co-expression network. The majority of upregulated transcripts were linked with cell cycle pathway regulation, including DNA replication and cell cycle phase progression, while downregulated transcripts were associated with immune-related pathways comprising TNF, Wnt and MAPK signalling pathways.

A further study by unrelated investigators used five pooled samples in a microarray analysis, comprised of 10 endometriosis serum samples, 10 endometriosis-free serum samples, six paired eutopic with six ectopic endometrial samples and six control eutopic endometrial samples (Wang et al., 2016a,b). In serum from women with endometriosis, 1682 dysregulated lncRNAs were identified, whereas 1435 lncRNAs were dysregulated in the eutopic endometrium, compared to samples from disease-free controls. From these, 55 concordantly and 70 discordantly dysregulated lncRNAs were identified in both the serum and tissue analyses, 16 of which were validated by qRT-PCR in serum samples from 59 women with endometriosis and 51 controls (Wang et al., 2016a,b). There were eight lncRNAs which demonstrated a significant difference in expression between women with and without endometriosis and one showed increasing dysregulation with disease progression. Serum levels of lncRNA ENST00000482343 showed the highest diagnostic test accuracy for endometriosis with a sensitivity of 72.41% and specificity of 71.74% (Wang et al., 2016a,b). This is not sufficient to reach the criteria for a replacement or triage test as defined by recent Cochrane reviews (Gupta et al., 2016; Nisenblat et al., 2016). However, this lncRNA is a likely contender for a combination test integrating multiple endometriosis biomarkers.

Together, these studies have identified many lncRNAs that may prove to be instrumental in the disease process. However, variation between study outcomes again implies a lack of sufficient power to draw firm conclusions. Although this was partially addressed by Wang et al. (2016a,b) through the use of single pooled samples, a
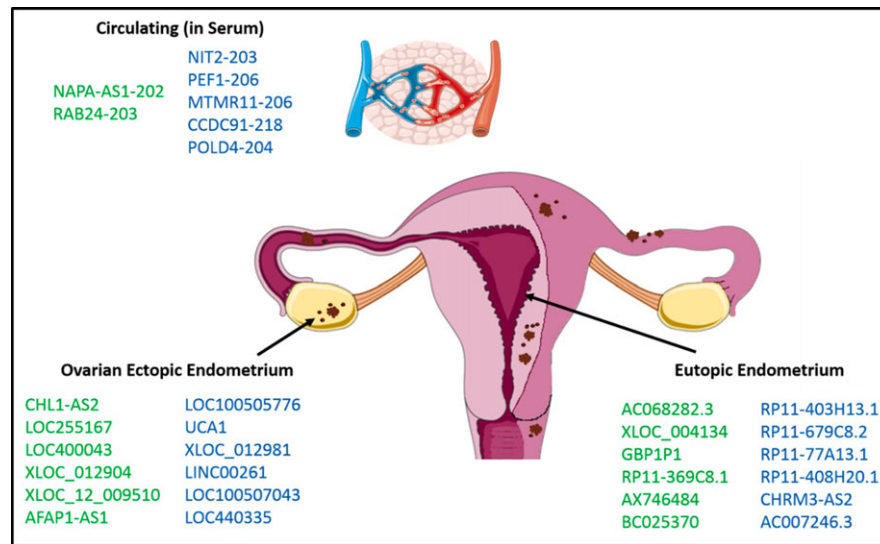
Figure 3 Long non-coding RNAs identified as dysregulated in endometriosis. The expression of multiple lncRNAs are altered in the circulating serum of women with endometriosis compared to healthy women and in the eutopic endometrium of women with endometriosis compared to healthy women, as well as in ovarian ectopic endometrium compared to eutopic endometrium from women with endometriosis. Upregulated lncRNAs are shown in green; downregulated lncRNAs are shown in blue.

large well-powered transcriptomic study has yet to be undertaken (Wang *et al.*, 2015, 2016a,b). Two studies have sought to validate the lncRNAs identified as dysregulated in endometriosis by qRT-PCR (Sun *et al.*, 2014; Wang *et al.*, 2015) but the result is questionable since in both cases the data were normalized to expression of *GAPDH* mRNA, an approach appropriate for mRNA but not miRNA. Others have used a more robust normalization method enabling absolute quantification (Wang *et al.*, 2016a,b) which sets a standard for future studies.

Short interfering RNAs

Short interfering RNAs (siRNAs) are ~22 nt long molecules cleaved in the cytoplasm from long double-stranded (ds) RNA by the RNAse-III enzyme Dicer (Vazquez and Hohn, 2013). The long dsRNA from which siRNAs originate can be generated endogenously by convergent and bidirectional transcription, by transcription of repeat sequences, by transcription of RNA in hairpin structures (Luo *et al.*, 2016) or from cleavage of mRNAs paired to antisense pseudo-gene transcripts or lncRNAs. Exogenous long dsRNA can also be introduced into cells by viruses, transfection and other laboratory manipulations (Bartel, 2005).

siRNAs bind in a perfectly complementary way to the sequence of their mRNA target, which is then cleaved and eliminated, with few off target effects. It is thought that plants and invertebrate animals without antibody or cell-mediated immunity use siRNA-mediated RNAi to protect their genome from dsRNA of viruses and other pathogens (Andersen and Panning, 2003; Hannon *et al.*, 2006; Luo *et al.*, 2016). As dsRNA generates an interferon-mediated immune response in mammals, endogenously generated siRNAs are thought to not participate in human biological processes, however, this remains unclear as endogenous siRNA have been described in cultured human cells (Yang and Kazazian, 2006).

Synthetic siRNAs are a potent tool for silencing expression of specific genes and this methodology has progressively advanced our knowledge of genes contributing to the endometriotic disease process. Additionally, this approach sheds light on the possible therapeutic utility of modulating specific genes using ncRNA strategies.

Utility of siRNAs in endometriosis

A shortcoming of many endometriosis studies is that siRNA have often been transfected into either primary or immortal monocultures of cells that do not adequately represent the structural complexity and cellular composition of endometriosis lesions. In particular, it is difficult to recapitulate the contribution of resident immune cells in *ex-vivo* models. A thorough knowledge of the cellular expression of the siRNA transcript target in endometriotic lesions is therefore required to ensure that the siRNA is evaluated in the appropriate cell lineage. Several studies have demonstrated the utility of siRNA-mediated gene silencing approaches in endometriosis research and have identified several potential therapeutic targets.

Regulation of migration
Jiang *et al.* (2012) demonstrated that increased *EZRIN* mRNA levels in ectopic endometrial cells correlate with increased Rho and ROCK GTPase-mediated cell migration. When an *EZRIN* silencing siRNA was transfected, reduced RhoA and ROCK1 expression was associated with inhibition of migration in ectopic endometrial cells (Jiang *et al.*, 2012). It was concluded that therapeutics that target Ezrin, Rho or ROCK might suppress the migration and adhesion of ectopic endometrial cells, to regulate disease development.

Regulation of macrophage activity

Macrophage numbers are elevated in the peritoneal fluid and ectopic tissues of women with endometriosis and these cells clearly influence endometriotic lesion development (Bacci et al., 2009; Capobianco and Rovere-Querini, 2013; Ahmad et al., 2014; Králíčková and Vetvicka, 2015). M1 inflammatory macrophages act to suppress, whereas M2 macrophages help to promote, tissue remodelling and enhance endometriosis-like lesion development in mice (Bacci et al., 2009). Strategies utilizing siRNAs have also demonstrated the importance of macrophages in endometriosis.

Chuang et al. demonstrated that peritoneal macrophages from women with endometriosis have reduced phagocytic ability, coupled to lower scavenger receptor CD36 expression, which together would be expected to reduce ectopic tissue clearance (Chuang et al., 2009). siRNA knock-down of CD36 resulted in impaired phagocytic ability in normal macrophages (Chuang et al., 2009), thus revealing a mechanism of immune dysfunction that could contribute to the pathogenesis of endometriosis.

When primary ESCs were co-cultured with M2 macrophages, STAT3 was upregulated in association with increased ESC proliferation. When siRNA inhibiting STAT3 was introduced into ESCs, their proliferation was suppressed, indicating that STAT3 activation participates in the dialogue between M2 macrophages and ESCs in endometriosis-like lesions and so may be a potential therapeutic target for disease modulation (Bacci et al., 2009; Capobianco et al., 2011; Capobianco and Rovere-Querini, 2013; Itoh et al., 2013).

Regulation of inflammatory pathways

In the chick embryo model of endometriosis, siRNA knock-down of nuclear factor-kappa beta (NFKB) in human eutopic endometrial fragments reduces vascularization of the chick chorioallantoic membrane and enhances apoptosis in ectopic tissues (Liu et al., 2009). These findings suggest that the inflammatory NFKB pathway primes ectopic endometrial cell survival and angiogenic pathways that are critical for the establishment of endometriosis lesions.

Regulation of cellular proliferation and apoptosis

Increased cellular proliferation, coupled with upregulation of anti-apoptotic gene expression is evident in early phases of in vivo models of ectopic endometrial lesion (Grümmer et al., 2001, 2012; Hull et al., 2008) and is seen in eutopic and ectopic endometrial tissues from women with endometriosis. siRNA knock-down experiments have identified genes with critical roles in allowing aberrant cell survival in endometriosis, revealing potential targets for therapeutic intervention.

Increased levels of leptin have been observed in the peritoneal fluid of women with endometriosis (Milewski et al., 2008; Alviggi et al., 2009) and leptin was found to stimulate the growth of human endometriotic epithelial cells by inducing STAT3, Janus Kinase 2 (JAK2) and ERK pathways (Oh et al., 2013). siRNA-mediated suppression of the leptin receptor (ObR) (Oh et al., 2013) reduces cellular proliferation in vitro. This raises the prospect that inhibiting leptin receptor activity in women might reduce endometriotic tissue load and disease progression.

In primary ESCs from endometriomas, siRNA transfection was utilized to silence the DR5 gene, a pro-apoptotic receptor of tumour necrosis factor-related apoptosis inducing ligand (TRAIL) (Hasegawa et al., 2009). TRAIL receptor (DR5) expression was effectively reduced, leading to inhibition of TRAIL-induced apoptosis (Hasegawa et al., 2009). Thus, impaired DR5 activity, or perhaps reduced bio-availability of the TRAIL ligand which feasibly could occur due to fewer cytotoxic immune cells that normally express this factor, might contribute to endometriotic cell survival and promoting disease development. Agents that enhance DR5 signalling activity and overcome the myriad biological controls on TRAIL-induced apoptosis (Mert and Sanlioglu, 2017), and/or enhance the activity of cytotoxic lymphocytes capable of TRAIL-mediated killing, may have a positive therapeutic benefit in endometriosis.

Regulation of angiogenesis and neurogenesis

VEGF-C promotes vascular permeability and endometriotic lesion development. Following VEGF-C targeted siRNA transfection, endothelial cell migration, angiogenesis and lesion development was significantly inhibited (Xu et al., 2013), suggesting another potential pharmaceutical target for endometriosis therapeutics.

Chen et al. (2014) have demonstrated the importance of the β-nerve growth factor (β-NGF) in a rat model of endometriosis. Targeted silencing of β-NGF using siRNA resulted in growth suppression in ectopic endometriotic implants. Additionally, a reduction in sympathetic and sensory nerve fibre density coupled with a significant improvement in generalized hyperalgesia was observed (Chen et al., 2014). These observations suggest that further evaluation of strategies to impair nerve growth and function is warranted, as an approach to limit pain symptoms in endometriosis.

Regulation of progesterone activity

siRNA have been used to silence the isoform B progesterone receptor (PR-B), resulting in increased proliferation of an immortalized human ESC line (Wu et al., 2008). The low numbers of PR-B receptors in the ectopic and eutopic endometrium of women with endometriosis (Igarashi et al., 2005) might therefore contribute to the increased proliferation and resistance to apoptosis seen in endometrial tissue in women with endometriosis. Further work needs to be done to assess downstream changes in gene expression after PR-B gene silencing to identify the biological pathways involved, and to determine the role of PR-B in disease progression and progesterone-resistance.

FKBP4, is a progestin receptor co-chaperone protein that is reduced in the endometrium of women with endometriosis (Yang et al., 2012). Transfection of ESCs with siRNA targeting FKBP4 resulted in reduced synthesis of insulin-like growth factor binding protein 1 (IGFBP1) (Yang et al., 2012). In endometriosis, low FKBP4 and a subsequent decline in IGFBP1 (a marker of decidualisation) may contribute to impaired decidualisation and subsequent infertility (Yang et al., 2012).

Clinical applications of ncRNA

Identification and experimental manipulation of ncRNAs has widened our understanding of many diseases, providing a rationale for exploring the potential of ncRNAs as biomarkers and therapeutic agents. As our comprehension of their function develops, clinically translatable applications for ncRNAs are now being developed. In illnesses such as myocardial infarction (Bye et al., 2016), consistently measureable differences in

miRNA levels raise the possibility that ncRNA dysregulations could be exploited as biomarkers for diseases such as endometriosis. Furthermore, the FDA has approved prostate cancer gene 3 lncRNA as a prostate cancer biomarker in urine (De Luca *et al.*, 2016). Initial studies have identified miRNAs and lncRNAs with potential as circulating biomarkers for endometriosis (Gupta *et al.*, 2016; Nisenblat *et al.*, 2016), however, there remains a need for larger transcriptomics studies involving more diverse patient cohorts (Rogers *et al.*, 2016). Efforts have been made to ensure uniformity in specimen collection and processing (Fassbender *et al.*, 2014; Rahmioglu *et al.*, 2014a) and to encourage the deposition of genomic profiling data in repositories (Pereira *et al.*, 2014; van Schaik *et al.*, 2014). Ideally, a collaborative global database of miRNA and lncRNA expression levels in tissues and blood of women with endometriosis and non-diseased controls would inform future biomarker initiatives. A patent was recently filed to utilize leucocyte miRNAs for the diagnosis and treatment of endometriosis, demonstrating progress in the commercial development of non-invasive diagnostic tools for endometriosis (Nagarkatti *et al.*, 2015).

Many research groups and commercial companies have explored the clinical translation of RNA-based therapeutics. To date only Macugen, a RNA aptamer which antagonizes VEGF has been approved by the FDA for the treatment of macular degeneration via intravitreal injection (Sankar *et al.*, 2016). RNA stability has been a barrier to successful development of RNA therapeutics but various approaches have been taken to address this limitation (Raja *et al.*, 2015; Abdelrahman *et al.*, 2017). The route of administration (e.g. oral, subcutaneous and intravenous) is another challenge. This must confer protection from RNase degradation, while supporting suitable distribution and persistence, with a sufficiently low elimination half-life to be clinically useful. Another hurdle in therapeutic RNA development is the need for careful consideration of treatment efficacy, delivery and potential side-effects and toxicity in humans. As exogenous ncRNAs modulate multiple mRNAs, dangerous off-target side effects or undesired immunologic responses could occur, which may only be noticeable in large cohort trials.

Advances in RNA modification, including locked-nucleic acid technologies, as well as delivery systems involving liposomes and nanoparticles, have facilitated human clinical trials of antagomirs, miRNA mimics, miRNA sponges, aptamers and siRNAs. Elmen *et al.* (2008) were the first to demonstrate the efficacy of miRNA-mediated silencing in non-human primates. Intravenous delivery of a locked nucleic acid modified oligonucleotide (LNA-antimiR) antagonized hepatic miR-122, reversibly decreased plasma cholesterol levels without toxic side effects or histopathological changes in African green monkeys. Alnylam Pharmaceuticals is currently undertaking a Phase III trial (NCT01960348) using the drug Patisiran (ALN-TTR02) which is a lipid nanoparticle containing siRNA that inhibits hepatocyte-driven transthyretin (Suhr *et al.*, 2015). To treat liver fibrosis, Phase I trials (NCT02227459) have been undertaken using the drug ND-L02-S0201 which targets the collagen-specific HSP47 chaperone protein involved in collagen biosynthesis (Nair *et al.*, 2016). Uniquely, these liposomes are coupled with vitamin A, and only hepatic stellate cells can uptake the delivered siRNA. The recently developed LODER (Local Drug EluteR) cancer drug delivery platform by Silenseed enables direct insertion of RNA treatments into tumour cores to sustain their therapeutic release over several months (Shemi *et al.*, 2015). Phase I trials for pancreatic cancer targeting KRAS using siG12D LODER (NCT01188785) were completed successfully in 2014, and Phase II trials (NCT01676259) are in progress. Recent studies utilizing a bacterial cell wall preparation equipped with tissue-specific targeting function has been demonstrated to be effective in delivering miRNA to tumour tissues in humans (van Zandwijk *et al.*, 2017).

Although no therapeutic RNA trials have been undertaken in gynaecology, these exciting clinical advances in other disciplines indicate the promise of ncRNA therapeutics for endometriosis. As endometriosis is considered to reflect an underlying systemic disorder (Naqvi *et al.*, 2016), intravenous delivery to treat multiple lesions as well as potentially circulating immune cells simultaneously may be desirable. Alternatively, ncRNA treatments could be directly administered into the peritoneal cavity. An approach such as that in the LODER delivery platform, which enables sustained release of ncRNA treatments, may allow sustained treatment of this recurrent, chronic disease. A challenge with RNA therapies is their potential to target multiple mRNAs in many different organ systems, which could result in off-target effects and adverse outcomes. To combat this, methods to target specific cells such as tagging hepatocytes or macrophages are in development. Targeting ncRNA pathways that regulate the phenotype and activation profiles of macrophages, lymphocytes or related immune cell subsets may be a useful avenue in the treatment of endometriosis, where these immune cells are critical for lesion development.

Conclusion and future directions

In endometriosis, there is ample evidence that miRNAs and lncRNAs regulate key cellular pathways in disease development and progression. However, defining the precise nature of their functions and significance will require higher quality studies to accommodate the complex, heterogeneous manifestation and symptoms of endometriosis that contribute to the enormous challenge of understanding this disease. Dynamic epigenetic changes in healthy endometrium have been mapped across the menstrual cycle (Rekker *et al.*, 2013), and by comparing this ncRNA profile with eutopic endometrium from women with endometriosis, it seems possible that reliable molecular differences in endometrial tissue might be identified to further our understanding of disease pathogenesis. However, clearly this will require large and well-curated cohorts with well-defined clinical data.

The recent expansion in awareness and growing community demand for investment in solving endometriosis has increased the imperative for novel research approaches, as well as the opportunity to assemble and interrogate large patient cohorts. Adolescents with early onset menarche have been identified as an at-risk population for developing a more severe disease (Ballweg, 2004; Matalliotakis *et al.*, 2017), and a New Zealand initiative for menstrual health education programmes in secondary schools (Bush *et al.*, 2017) has shown increased awareness of endometriosis and earlier presentation of affected young women to gynaecologic specialists. However, as a surgical diagnosis of endometriosis is still required, it is essential that identification of sensitive and specific non-invasive disease biomarkers is a top priority. Several commercial initiatives are underway to develop a diagnostic tool for endometriosis that incorporates ncRNAs. However, the variation in experimental design and sample collection methods from women with endometriosis is a limitation that has hindered the accurate and consistent identification of aberrant ncRNA expression between cohorts.

Moving forward, to fully exploit the potential of ncRNAs as non-invasive diagnostic markers, the need remains for larger genome-wide profiling studies involving a more diverse but clinically well-characterized patient cohort to be carried out. Ideally, establishing a global collaborative database of ncRNA expression levels in both disease and non-diseased conditions would be essential to ensure uniformity in specimen collection and processing methodologies. In addition, due to the multi-factorial nature of endometriosis, rather than using a single biomarker, it may increase predictive capacity and diagnostic accuracy to employ a panel of ncRNA biomarkers.

Functional outcomes of RNA-mediated gene silencing using siRNA technology in *in vitro* experiments, and initial *in vivo* experiments have been informative and warrant further investment. Moreover, the availability of small animal models with miRNA deficiencies, as well as advancements in CRISPR and CRE-LoxP genome engineering and editing technology, should be exploited. Such approaches to evaluating the impacts of ncRNA overexpression or genetic deficiencies in vivo are likely to be informative on the specific pathophysiological roles of individual miRNA, and to identify aspects of the disease process that are vulnerable to intervention.

Collectively, technological advances in sequencing methodologies and, in *in vitro* and *in vivo* ncRNA delivery systems will continue to advance understanding and underpin progress in this important field. The advent of simple yet robust assays and bioinformatic analysis software will further increase the predisposition for clinical labs and research groups to routinely evaluate ncRNA expression levels in various samples. As our knowledge of ncRNA in endometriosis expands, we will gain a clearer understanding of the clinical applicability of their use in diagnosing and potentially in treating this important and challenging disease.

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Authors' roles

K.P. formulated the primary draft of the paper and primarily undertook literature searches, data analysis and interpretation. M.L.H. supervised the conception, design and development of all aspects of the article and was involved in the data analysis and interpretation. Development of the article, review and editing of the final article was by K.P., J.E.S., S.A.R. and M.L.H.

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Conflict of interest

The authors have no conflict of interest to declare.

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