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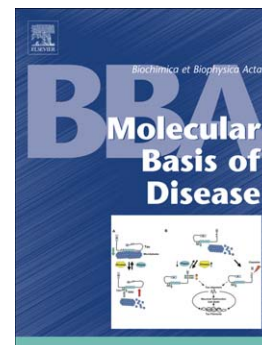
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Challenges in endometriosis miRNA studies - from tissue heterogeneity to disease specific miRNAs

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Abbreviations

EcSC - endometriotic stromal cells

EMT - epithelial-mesenchymal transition

EnSC - endometrial stromal cells

FACS - fluorescence-activated cell sorting

FC - fold change

LCM - laser capture microdissection

UTR – untranslated region

ABSTRACT

In order to uncover miRNA changes in endometriosis pathogenesis, both endometriotic lesions and endometrial biopsies, as well as stromal and epithelial cells isolated from these tissues have been investigated and a large number of dysregulated miRNAs has been reported. However, the concordance between the result of different studies has remained small. One potential explanation for limited overlap between the proposed disease-related miRNAs could be the heterogeneity in tissue composition, as some studies have compared highly heterogeneous whole-lesion biopsies with endometrial tissue, some have compared the endometrium from patients and controls, and some have used pure cell fractions isolated from lesions and endometrium. This review focuses on the results of published miRNA studies in endometriosis to reveal the potential impact of tissue heterogeneity on the discovery of disease-specific miRNA alterations in endometriosis. Additionally, functional studies that explore the roles of endometriosis-involved miRNAs are discussed.

1. Introduction

Although several hints about biological processes leading to endometriosis development have emerged in the era of high-throughput studies, we still lack in-depth knowledge on the exact molecular basis of the disease development. Without unravelling all the components that play a role in different molecular processes of endometriosis pathophysiology, it is impossible to solve the mystery of this disease and to find a definite cure.

In endometriosis, the female internal reproductive system and other pelvic organs are affected by endometriotic lesions growing on the surface of pelvic cavity organs (ovaries, uterus and fallopian tubes), ligaments (uterosacral ligaments, broad and round ligaments), recto-uterine pouch and/or ovarian fossa. During laparoscopic surgery, lesions are removed and these tissue samples are often used in endometriosis studies to find disease-specific changes. However, the cellular composition of whole-lesion biopsies is heterogeneous and contains cells from surrounding tissue (e.g. peritoneal tissue, ovarian components, traces of uterine wall etc.), inflammatory cells, and endometrial stromal and epithelial cells in variable proportions. Therefore, the heterogeneity of endometriotic lesion biopsies is a real challenge in endometriosis research, as the molecular signature of endometrial cells in lesions could be masked by the surrounding tissue leading to inconsistent or wrongly interpreted results.

microRNAs (miRNAs) are small (typically 22 nucleotides in size) non-coding RNA molecules that regulate target mRNA expression. The most common mechanism of action involves an interaction of the so-called seed-sequence of the miRNA with the 3'UTR of target mRNAs, resulting in translational inhibition, mRNA degradation and/or destabilisation [1]. Therefore, changes in the dynamic balance between miRNAs and their target mRNAs may lead to alterations in normal physiological status of the tissues and initiate pathological processes. The knowledge about the miRNA signatures in tissues is constantly evolving and curated in databases presenting tissue-specific miRNA profiles (e.g. miRmine - Human

miRNA Expression Database [2], miRGator [3], the Human miRNA Tissue Atlas [4]), showing that miRNA levels in different tissues are highly variable. Furthermore, tissue-specific miRNA expression levels are also influenced by normal physiological processes, such as endocrine influences throughout the menstrual cycle in endometrium [5].

A number of miRNAs have been reported as dysregulated in endometrium and lesions of endometriosis patients [5–7]. However, different studies have not reached a consensus on which particular miRNAs are most relevant in endometriosis, as dysregulated miRNAs reported in one study are only occasionally confirmed by others. In our previous miRNA study [8], we showed that miRNA profile of peritoneal endometriotic lesion biopsies is more similar to healthy peritoneal tissues rather than to endometrium of the same women. We also showed that endometriotic lesion biopsies contain only a small fraction of endometrium-specific cells and various proportions of non-diseased tissue that can mask the disease-specific miRNA changes in endometriotic lesions. Therefore, we suggest that one potential explanation for limited overlap between the proposed disease-related miRNAs in endometriosis is heterogeneity in tissue composition, as some studies have compared whole-lesion biopsies with endometrial tissue, some have analysed endometrial tissue from patients and controls, and some have used pure cell fractions isolated from lesions and endometria (Figure 1).

In this review, we focus on the results of published miRNA studies in endometriosis to discuss the potential impact of tissue heterogeneity on the discovery of disease-specific miRNA alterations in endometriosis. We believe that the question about the tissue biopsy heterogeneity needs to be highlighted and not only in endometriosis molecular research but also in many other diseases where heterogeneous tissue biopsies have been used to find disease specific alterations. Also, we give an overview of functional studies performed to find

out the precise roles of miRNAs in endometriosis pathogenesis, and propose future directions in endometriosis miRNA research.

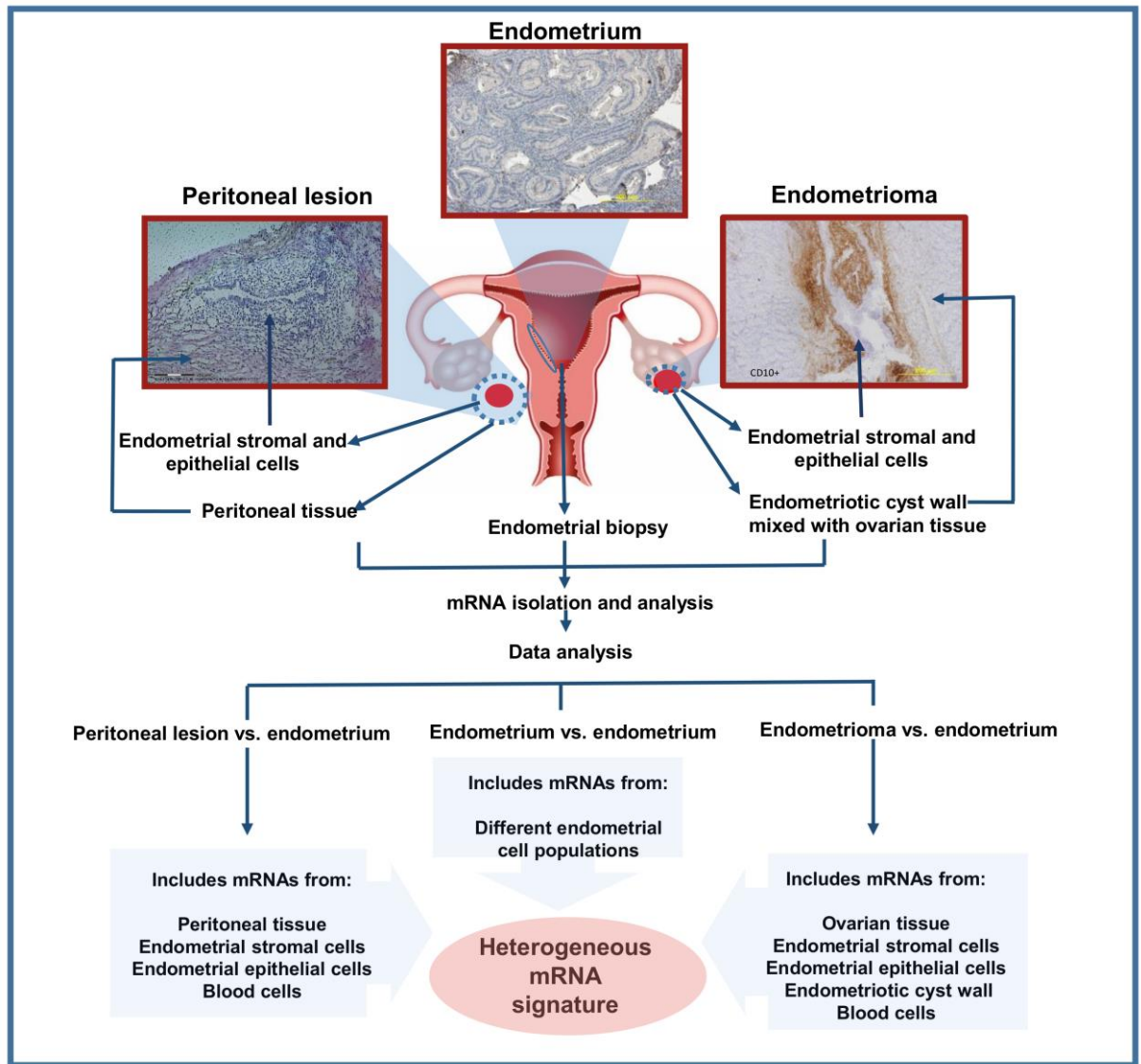


Figure 1. Origin of tissue heterogeneity in endometriosis miRNA studies. Peritoneal lesions and endometriomas contain only a small fraction of endometrium-specific cells leading to the heterogeneous miRNA signature (brown colour in endometrioma histological section indicates CD10+ endometrial stromal cells that surround the endometrial epithelial glandular structure).

2. High tissue/cellular heterogeneity - miRNA expression profiling studies in endometriotic lesions

A large number of published studies have evaluated associations between miRNAs and endometriosis by comparing miRNA expression levels in endometrium (consisting mostly of epithelial and stromal cells) to lesions that additionally include surrounding tissue (peritoneal or ovarian tissue) in variable proportions (Table 1). Both hypothesis-driven studies, investigating dysregulation of particular miRNAs [9–14] and high-throughput studies [8,15–20] have been used to find lesion-specific disease-related changes in miRNA levels (Table 1). More detailed information about the results of these studies and the potential roles of dysregulated miRNAs is available in recently published excellent reviews [7,21]. A closer look at high-throughput studies, where the full spectrum of miRNAs expressed in tissues were studied [8,15–20], indicates that the number of dysregulated miRNAs potentially associated with endometriosis pathogenesis is relatively variable (5 to 156 miRNAs per study). Moreover, in perspective of tissue type heterogeneity, three of these studies investigated endometriomas [16,17,20], two endometriotic lesions with no specification [18,19] and one study included peritoneal lesions [15]. Venn analysis (data not shown) of all found differentially expressed miRNAs between lesions and endometrium (Fold Change - FC at least 1.5), irrespective of the analysed lesion location and without taking into account the miRNA strand from which it was derived (-5p or -3p), showed only a small overlap between the result of these six studies. Fourteen miRNAs were significantly differentially expressed in at least three studies (Table 2) and only miR-200b was found to be differentially expressed in all six studies. However, it should be pointed out that none of the aforementioned high-throughput studies included information on whether multiple testing correction was implemented in data analysis, and therefore some of the reported associations may also be false-positive.

Table 1. High tissue/cellular heterogeneity - miRNA studies comparing endometriotic lesions to endometrium.				
Candidate miRNA studies				
miRNAs	Studied tissues		Menstrual cycle phase*	References
	Patients (n)	Controls (n)		
miR-15b, miR-16, miR-17-5p, miR-20a, miR-21, miR-125a, miR-221 and miR-222	Endometriomas (n=41) Endometrium (n=41)	Endometrium (n=38)	P/S	[9]
miR-191	Endometriomas (n=12)	Healthy ovaries (n=12)	ND	[10]
miR-451	Peritoneal lesions and endometriomas (n=43) Endometrium (n=30)	-	ND/M/P/S	[11]
miR-23a and miR-23b	Endometriomas (n=23) Endometrium (n=23)	Endometrium (n=15)	ND	[12]
miR-29a, miR-29b and miR-29c	Lesions (n=20) Endometrium (n=20)	Endometrium (n=10)	P	[13]
miR-143, miR-145	Endometriomas (n=22) Endometrium (n=13)	Endometrium (n=22)	P/S	[14]
High-throughput studies				
High-throughput methodology	Studied tissues		Menstrual cycle phase	References

	Patients (n)	Controls (n)		
Custom-made microarray	Peritoneal lesions (n=7) Endometrium (n=7)	-	P/S	[15]
Custom-made microarray	Endometriomas (n=3) Endometrium (n=3)	-	P	[16]
Illumina next-generation sequencing	Endometriomas (n=10)	Endometrium (n=9)	P/S/I	[17]
miRCURY LNA™ microRNA Array (v. 14.0; Exiqon)	Lesions (n=3) Endometrium (n=3)	Endometrium (n=3)	ND	[18]
Illumina next-generation sequencing	Peritoneal lesions (n=5) Peritoneum (n=4) Endometrium (n=2)	-	P/S	[8]
Affymetrix GeneChip miRNA array	Lesions (n=5)	Endometrium (n=5)	ND	[19]
Affymetrix GeneChip miRNA 2.0 Array	Endometriomas (n=3) Endometrium (n=7)	Endometrium (n=5)	P/S	[20]

*M - menstrual P -proliferative, S - secretory, I - interval, ND - menstrual cycle phase is not determined

Table 2. Common miRNAs found to be differentially regulated in endometriotic lesions compared to eutopic endometria.

miRNA	Number of studies (n)	Upregulated/downregulated in lesion	Reference
miR-200b	6	Downregulated	[15–20]
miR-145	4	Upregulated	[15,16,19,20]
miR-196b	4	Downregulated	[15,16,18,20]
miR-200c	4	Downregulated	[16–18,20]
miR-200a	4	Downregulated	[15–17,20]
miR-183	3	Downregulated	[16,18,20]
miR-202	3	Upregulated	[16,18,20]
miR-141	3	Downregulated	[15,17,20]
miR-34c	3	Downregulated	[15,17,20]
miR-100	3	Upregulated	[15–17]
miR-375	3	Upregulated	[16,17,20]
miR-1	3	Upregulated	[15,16,20]
miR-365	3	Upregulated/downregulated	[15,16,20]
miR-29c	3	Upregulated	[15,16,20]

Furthermore, although the overlap between studies is as scarce as it is, even so it may be overestimated as nomenclature of miRNAs in these studies is not uniform and not all studies have referred to the examined miRNAs using the strand-specifying -3p and -5p suffixes or other identifiers (e.g. miRBase MIMAT accession number or oligonucleotide sequence). The importance of proper miRNA referring has previously been accentuated [7], as the expression levels of miRNAs generated either from -5p or -3p arm of the precursor may vary not only between different tissues/cells but also in health and disease [22].

The most frequently reported differentially expressed miRNA, miR-200b, belongs to the miR-200 family that could easily be linked to endometriosis pathogenesis because of involvement in cell migration and epithelial-mesenchymal transition (EMT). A comprehensive literature review concerning EMT, basic cellular pathways and its importance in endometriosis has been published recently [23]. Briefly, the miR-200 family members target a complex network of transcription regulators like *ZEB1* and *ZEB2* (E-box-binding transcription factors 1 and 2, respectively), which are transcriptional repressors for E-cadherin. The overexpression of miR-200 family members results in decreased expression of *ZEB1/ZEB2* and increased expression of E-cadherin, which is required for maintaining the epithelial nature of cells [24]. The epithelial cell plasticity is controlled by an autocrine TGF- β /*ZEB*/miR-200 signalling network [25] and by the Wnt/ β -catenin pathway (reviewed in [23]). During the EMT process, which supposedly occurs in the pathogenesis of endometriosis, epithelial cells lose their specific features and integrity, and acquire mesenchymal characteristics, leading to increased cell invasion and migration [24]. All aforementioned miRNA studies in endometriosis have confirmed significant downregulation of miR-200b in endometriomas (~2-fold) and peritoneal lesions (2.8-fold) compared to endometrium. Four studies have also reported downregulation of other miR-200 family members miR-200a and miR-200c, and three studies downregulation of miR-141 in lesions

(Table 2). Although all studies in endometriotic lesions have associated miR-200 family members to disease pathogenesis, we have shown that in healthy endometrium miR-200a, miR-200b and miR-141 are highly expressed in epithelial cells, indicating the cell type-specific expression pattern of these miRNAs [8]. Therefore, it is possible that the reported seemingly lower expression of miR-200 family members in endometriotic lesions reflects the smaller proportion of epithelial cells in lesions rather than is associated with disease pathogenesis. Thus, the true relevance of miR-200 family in endometriosis pathogenesis needs to be confirmed in the future by comparing pure populations of eutopic and ectopic endometrial epithelial cells. Interestingly, a very recent study investigating miRNA expression in fluorescence-activated cell sorting (FACS) isolated eutopic endometrial cells of patients with and without endometriosis [26] showed higher expression of miR-200b in stromal cells of patients compared to controls but there was no difference in miR-200b expression in respective epithelial cells (the results of this study are more thoroughly discussed in Section 4).

The second miRNA commonly found upregulated in endometriotic lesions is miR-145 (miR-145-5p [15,16,19] and miR-145-3p [20]). This miRNA promotes apoptosis in growing cancer cells and inhibits cancer cell invasion and metastasis (reviewed in [27]); however, the importance of miR-145 in colon cancer pathogenesis has been recently questioned [28]. miR-145 is highly expressed in mesenchymal cells such as fibroblasts and smooth muscle cells, and not in colon cancer or healthy colon epithelial cells; therefore, the lower expression in cancer tissue compared to healthy colon merely reflects the differences in cellular composition of tissues and not miR-145 dysregulation in cancer cells [28]. The situation may be similar in endometriosis, as endometriotic lesion biopsies contain a mixture of different cells, including cells from surrounding tissue (e.g. ovarian stroma and peritoneal mesenchymal cells) that are not present in eutopic endometrium, and thus we cannot exclude

that the higher miR-145 level in lesions just reflects the cellular heterogeneity of the tissue sample. Still, *in vitro* studies using endometriotic and endometrial stromal cells have revealed a role for miR-145 in the regulation of invasive growth and a stem cell phenotype, as will be discussed in Section 5.1 [29].

The third most commonly dysregulated miRNA in lesions is miR-196b, with a genomic location between the *HOXA9* and *HOXA10* genes. It should be pointed out that miR-196 family miRNAs, miR-196a and miR-196b, target many genes from the HOX cluster e.g. *HOXA5*, *HOXB7*, *HOXB8*, *HOXC8* and *HOXA10*. The latter has been well described in the regulation of the endometrial function and there is clear evidence that the pattern of *HOXA10* expression depends on menstrual cycle phase, with a peak expression during the window of implantation [30]. Considering the role of miR-196b in tissue vascularization and wound healing in response to injury or other pathological conditions [31], and also its possible role in normal endometrial physiology, this miRNA has good potential to be related to endometriosis pathogenesis or endometriosis-associated infertility. How the magnitude of vascularisation in lesions and endometrium, and the menstrual cycle phase could influence the levels of miR-196b, needs to be clarified in further studies.

Taken together, although the miRNA studies in whole lesion biopsies have brought out some promising miRNAs that could be involved in endometriosis pathogenesis, the question about the impact of cellular heterogeneity on these findings remains. The remarkably higher cellular heterogeneity of lesions compared to endometrium may have considerable effect on the detection rate of differentially expressed miRNAs. To overcome this issue, future studies looking for differences in miRNA levels should focus on the isolation and analysis of the endometrium-specific cells inside the lesions instead of using whole lesion biopsies. Possible approaches to achieve this goal are described in the next section (Section 2.1).

2.1 Possible approaches to diminish cellular heterogeneity in endometriosis studies

Currently, one of the major challenges in endometriosis miRNA research is related to the isolation of specific cell-types from tissue specimens, as there is still no ideal methodology for obtaining pure populations of endometrial cells without cell culturing. Although miRNAs can be successfully isolated from histological tissue sections by laser capture microdissection (LCM) technique and isolated miRNAs are suitable for high-throughput analyses [32,33], the popularity of this methodology in endometriosis miRNA research has remained small. Though this methodology has been implemented to detect DNA aberrations [34,35] and gene expression alterations [36] in endometriosis, there are still no studies using LCM-isolated endometriotic cells to explore the endometriosis miRNome. The small interest of using LCM for isolation of specific cell populations could be related to the complexity and availability of this methodology.

The other possibility is to use combinations of fluorescently labelled antibodies against cell surface markers (for example, CD13 and CD10 have previously been shown to be markers of endometrial stromal cells, and CD9 is a marker of epithelial cells [37,38]) and FACS to isolate uncultured single cells or cell populations from tissues. FACS methodology has previously been used to isolate specific cell types from endometrium of healthy women [39] and from endometrium of women suffering from endometriosis [26,40]. We have successfully isolated CD10-positive stromal cells from whole lesion biopsies [40] for transcriptomic study, indicating that this methodology can be successfully used to study endometriotic lesions. The current limitation of this methodology in endometriosis research is the shortage of specific antibodies to discriminate endometrial epithelial cells from lesion biopsy. For instance, anti-CD10 antibody discriminates well CD10-negative cells ovarian stromal cells from endometrial CD10-positive cells stromal cells [38] but epithelial cell-specific anti-CD9

antibody that has been previously used for endometrial epithelial cell isolation [39] does not segregate ovarian CD9-positive cells granulosa cells [41] and epithelial cells from ectopic lesion. Also, it should be pointed out that beside endometrial epithelial and stromal cells, lesions contain also other cell types (endothelial cells etc.) that could have impact on disease pathogenesis. Therefore, computational methods that allow to infer cell-type specific expression profiles from whole-tissue samples, also known as gene expression profile deconvolution, can be used [42,43]. In conclusion, in spite of some shortcomings, these methodologies and ideas make it possible to move from highly heterogeneous whole-tissue approach to the cell type-specific approaches progressing endometriosis miRNA research to the next level.

3. Moderate tissue/cellular heterogeneity - miRNA studies in endometrium

Endometrium is a mixture of various cell types (e. g. stromal, epithelial, endothelial, immune cells) and the proportion of these cell types and the amount of blood vary in different endometrial biopsies, depending on the menstrual cycle phase and individual variability. Still, the cellular heterogeneity between endometrial samples from patients and controls is less pronounced than between endometrial and lesion biopsies, enabling to detect endometriosis-associated changes more reliably. Indeed, previous molecular profiling studies in eutopic endometrium have suggested that gene expression alterations, that facilitate proliferation, implantation and survival of endometrial tissue in peritoneal cavity, exist in endometrial cells of endometriosis patients [44] supporting the theory that endometriosis pathogenesis may partially originate from altered eutopic endometrium. To understand the possible role of miRNAs from eutopic endometrium in endometriosis development, a number of hypothesis-

driven and hypothesis-free studies have been performed. Hypothesis-driven studies focusing on single miRNAs have reported significantly lower expression of miR-199a [45], miR-126 [46], miR-23a, miR-23b [12], miR-29c [13], miR-451 [47], higher expression of miR-202 [48] and no significant changes of miR-143 and miR-145 [14] in endometrium of endometriosis patients compared to disease-free women (Table 3). Also, significantly higher levels of miR-135a and miR-135b were reported in eutopic endometrium of endometriosis patients; however, miR-135a was differentially expressed only in proliferative phase samples in patients with endometriosis compared to controls, indicating the impact of menstrual cycle phase on miRNA expression [49].

During the recent years, some hypothesis-free high-throughput miRNA studies using endometrial tissue of patients and controls have also been performed [18,20,50–52], Table 3. It should be pointed out that the number of differentially expressed miRNAs (FC at least 1.5) has been relatively moderate (altogether 132 miRNAs, ranging from 6 - 69 per study), indicating that the overall differences between the endometrial miRNA signatures of patients and controls are rather insignificant. Moreover, if we compared the results of different studies, there were no miRNAs, which had been reported in at least three studies. Interestingly, some alternatively expressed miRNAs described in hypothesis-driven miRNA studies, such as miR-23a, miR-23b, miR-29c and miR-145, have also been found to be altered in a microarray-based study [50].

During the menstrual cycle, endometrium undergoes cyclic growth and degeneration and the levels of some miRNAs change along with the normal endometrial physiology. For example, the expression of miR-29b, miR-29c, miR-30b, miR-30d, miR-31, miR-193a-3p, miR-203, miR-204, miR-200c, miR-210, miR-582-5p and miR-345 was higher in secretory than in proliferative phase endometrial epithelial cells [53] and miR-181, miR-183 and miR-200 family miRNAs were downregulated during the decidualization process [54].

Additionally, some miRNAs (miR-30b, miR-30d, and miR-494) may participate in the regulation of endometrial receptivity [55]. Therefore, at least some variability described at miRNA level in endometrium of endometriosis patients could be caused by menstrual cycle fluctuations if samples from different phases have been analysed together (Table 3). Moreover, in some studies, the respective information about menstrual cycle phases of collected tissues is missing.

To conclude, the question whether endometrial miRNAs contribute to the development of endometriosis has been tackled by many researchers, but the inconclusiveness and small overlap between the results of distinct studies suggests that either endometrial miRNA expression levels between patients and controls are relatively invariable and do not contribute to endometriosis development, or the differences in menstrual cycle phases, array platforms used, selection of patients and controls, data analysis and interpretation etc., mask the potentially important but minor changes in endometrial miRNA levels. Therefore, studies elucidating the role of miRNAs in regulation and function of normal endometrial physiology are warranted before any disease-related endometrial miRNAs can be found.

Table 3. Moderate tissue/cellular heterogeneity - miRNA studies comparing endometria from patients and controls

	Patients (n)	Controls (n)	Menstrual cycle phase*	References
Candidate miRNA studies				
miR-199a	12	12	P/S	[45]
miR-143, miR-145	13	22	P/S	[14]
miR-202	26	25	ND	[48]
miR-126	31	27	S	[46]
miR-23a, miR-23b	23	15	P	[12]
miR-29a, miR-29b, miR-29c	20	10	P	[13]
miR-135a, miR-135b	32	50	P/ES/MS/LS	[49]
miR-451	7	19	MS	[47]
High-throughput studies				
High-throughput methodology	Patients (n)	Controls (n)	Menstrual cycle phase*	References
miRCURY LNA microRNA Array, Exiqon	10	11	P	[50]
TaqMan Array Human MicroRNA	10	10	P	[52]
miRCURY LNA microRNA Array, Exiqon	4	3	ES	[51]
miRCURY LNA microRNA Array, Exiqon	3	3	ND	[18]
GeneChip miRNA 2.0 Affymetrix array	7	5	P/S	[20]

* P - proliferative, S - secretory, ES - early secretory, MS - mid secretory, ND - not determined.

4. Low tissue/cellular heterogeneity - miRNA studies in isolated and cultured cells

The isolation of endometrial tissue-specific cells from lesions without any contamination of the surrounding tissue is a big challenge. The most widely used approach to obtain pure cells of interest is cell culturing that enables to isolate endometrial stromal and epithelial cell fractions with minimal contamination of other cells. However, this approach has its own limitations, such as cell culturing-induced expression alterations and difficulties in culturing primary endometrial epithelial cells. Furthermore, one issue that needs to be addressed in case of endometriomas is possible contamination of primary endometrial stroma cell cultures with ovarian stroma. There are no straightforward methodologies to evaluate the amount of ovarian stromal cells throughout the cell passages [56] and using cell cultures that include a mixture of endometrial and ovarian cells in unknown proportions may influence the results of transcriptomic studies. Still, the analysis of cultured primary stromal cell originating from endometrium (EnSC) or ectopic lesions (EcSC) could be a useful and valuable tool for miRNA studies offering the lowest cellular heterogeneity, as the same type of cells, irrespective of original location, are compared.

To date, there is only one study comparing miRNA signatures of eutopic and ectopic stromal cells using high-throughput microarray approach. Twelve differentially expressed miRNAs were found [57], some of which were novel in the context of endometriosis (miR-214, miR-445-3p, miR-210 and miR-181a), while most have previously been reported in whole-lesion biopsy or endometrium studies. For example, miR-196b [15,16,18,20], miR-100 [15–17], miR-424-5p [15,20], miR-503, miR-199a-3p, miR-132* [16] were detected in whole-lesion biopsies and miR-29b [16,50,52] and miR-199b-5p [20,50] both in lesions and endometrium. However, as most of these miRNAs were not found to be dysregulated in endometrial tissues of endometriosis patients, it raises the question whether those expression differences precede lesion formation or are caused by the surrounding environment of

endometrial cells in ectopic location. Moreover, the endometrial expression levels of some of these miRNAs, such as miR-100, miR-424, miR-503 [58] and miR-199a [59] are influenced by the menstrual cycle phase. It can be hypothesised that the reason why these miRNAs were differentially expressed in lesions is hormonal dysregulation, as it is very likely that endometrial stromal cells in ectopic locations do not respond to hormonal changes in the same way as endometrial cells in uterus [60].

A different approach to obtain pure cell populations was used in a very recent study in which miRNA profiles of both FACS-isolated stromal and epithelial cells from endometria of patients with and without endometriosis were analysed [26]. The analysis revealed clear clustering of microarray data: firstly, according to cell type (stromal and epithelial) and secondly, according to the disease status (control and endometriosis). However, only 9 and 16 differentially expressed miRNAs were found in stromal and epithelial cells of patients compared to controls, respectively. Therefore, the analysis confirmed the results of whole endometrial tissue studies showing relatively few changes in endometrial miRNA expression levels in endometriosis. Interestingly, the most commonly described, miR-200b (downregulated in lesions) was found to be upregulated in stromal cells of endometriosis patients, and miR-375 (upregulated in whole-lesion biopsies) demonstrated lower expression in diseased epithelial cells [26]. Only two of these dysregulated miRNAs (miR-629 and miR-3136) were previously mentioned in a study comparing whole endometrial tissues of women with or without endometriosis [50].

To summarize, the small number of differentially expressed miRNAs in EcSC compared to EnSC indicates that the overall signature of miRNAs is relatively similar in endometrial stromal cells regardless of the origin. However, it cannot be ruled out that cell culturing has an impact on gene expression levels and thus, some miRNA alterations between eutopic and ectopic stromal cells may not be detected, as during the culturing cells may

undergo changes in transcriptomic profiles and lose their specific features. The transcriptome studies have shown that during cell culturing, a considerable shift in subpopulation dynamics will occur in response to *in vitro* growth conditions and thus the cell passaging may lead to selection pressure for specific cells inside the cell population (for example trypsin-sensitive cells, adherent cells, more active and proliferative cells etc.) that finally causes alterations in the overall transcription profile [61,62].

5. Functional studies in eutopic and ectopic endometrial cells in endometriosis

The most common application of EnSCs and EcSCs has been to explore the functions of miRNAs in stromal cells. Based on the function, studied miRNAs can be broadly divided into four categories: 1) miRNAs regulating apoptosis, 2) miRNAs involved in EMT, 3) miRNAs regulating cell proliferation, angiogenesis, invasion and viability and 4) miRNAs contributing to endometrial receptivity and infertility.

5.1 miRNAs regulating apoptosis

Properly functioning and strictly regulated apoptotic pathways are required to maintain organism homeostasis and therefore all abnormal fluctuations in this process could finally end up as potentially pathogenic. miRNAs are the key regulators of apoptosis by positive or negative regulation of anti- or pro-apoptotic mRNAs [63]. In endometriosis, the resistance to apoptosis has been considered as one possible cause why endometrial cells can survive in ectopic locations [64].

One of the anti-apoptotic miRNAs, miR-183, was found to be downregulated in eutopic and ectopic endometrium of endometriosis patients compared to control endometrium [16,18,20]. As this miRNA also regulates many other important cellular processes that are

considered to be involved in endometriosis pathogenesis, such as cell growth, differentiation, motility, cell adhesion and invasion [65,66], the role of miR-183 has been explored in this context. Functional analysis confirmed that miR-183 plays a promotional role in EnSC apoptosis and negatively regulates the invasiveness of cells, although it had no effect on EnSC proliferation [18]. Further, Chen et al. [67] verified that integrin β 1, a critical factor for cell adhesion and invasiveness, is a direct target gene of miR-183, and suggested that miR-183 may have negative regulatory impact on cell invasiveness and overexpression of integrin β 1 will block the repressive effects of miR-183 on EnSC invasiveness. Okamoto and colleagues [68] found that *STAT3*, an important regulator of several cellular processes, such as cell growth and apoptosis, was one of the key target molecules of miR-210. They suggested that anti-apoptotic miR-210 induces EnSCs to differentiate into the endometriotic phenotype, and that upregulation of miR-210 in EcSCs is involved in the formation of endometriosis-specific cellular dysfunctions [68]. miR-503 was found to be epigenetically repressed by hypermethylation in endometrial stroma cells from ovarian endometriotic tissues. Upregulation of miR-503 in these cells resulted in an inhibition of cell proliferation and induction of cell-cycle arrest at the G0/G1 phase through the suppression of cyclin D1, and the induction of apoptosis via suppression of the anti-apoptotic protein Bcl-2 [69].

The functional impact of anti-apoptotic miR-145 overexpression and inhibition on endometriotic cell behaviour and target gene expression was studied by Adammek et al. [29]. They showed that miR-145 inhibits endometriotic cell proliferation, invasiveness and stemness through regulation of cytoskeletal elements, cell adhesion molecules, proteolytic factors and pluripotency genes (*FASCIN-1*, *JAM-A*, *SERPINE1*, *CT4*, *ACTG2*, *TAGLN*, *SOX2*, *KLF4*, and *PODXL*).

Additionally, studies of pro-apoptotic miRNAs have shown the involvement of miR-195 and miR-29c in endometriosis pathogenesis. Higher expression of miR-195 in control

women EnSCs compared to patients' EnSCs and EcSCs was shown, and cell-to-cell adhesion and communication facilitating *FKN* was identified as a functional target of miR-195 [70]. Furthermore, miR-195 inhibits the expression of survivin and MMP9, and therefore, the lower level of this miRNA in endometrial cells in endometriosis may enhance EcSC survival in the peritoneal cavity [70]. The studies of miR-29c function in endometriosis pathogenesis revealed that miR-29c directly affects specific extracellular matrix genes in ovarian endometriomas [17], and alteration in miR-29c level may lead to suppression of endometrial cell proliferation and invasion and promote cell apoptosis via negative regulation of c-Jun expression [13].

5.2 miRNAs regulating epithelial mesenchymal transition

The dysregulation of miR-200 family (discussed in Section 2) in endometriotic lesions [8,15–20] has been associated with higher migratory capacity of the cells, as during the EMT, the epithelial cells lose their epithelial features and acquire motile mesenchymal phenotype. *In vitro* upregulation of miR-200b in EnSCs resulted in downregulation of *ZEB1* and *ZEB2*, and upregulation of E-cadherin, followed by decreased cellular invasiveness and motility [71]. In addition to regulating EMT and invasive cell behaviour, miR-200b upregulation in the endometriotic 12Z cell line also increased the expression of the transcription factor KLF4 and thereby enhanced endometrial cell proliferation and the stemness-associated side population phenotype *in vitro* [71].

In endometriosis, the role of miR-10b in EMT is still unknown, but it was shown in nasopharyngeal carcinoma cells that miR-10b regulates EMT through the KLF4/Notch1/E-cadherin pathway, promoting cell migration and invasion [72]. Recently, it was demonstrated that miR-10b and its predicted target Syndecan-1 inhibit epithelial endometriotic cell

invasiveness and viability through downregulation of metalloproteinase activity and IL-6 secretion [73].

5.3 miRNAs regulating cell proliferation, angiogenesis, invasion and viability

Many functional studies in EnSCs have focused on the miRNAs participating in the regulation of cell proliferation, angiogenesis, invasion and cell viability. A whole biopsy study revealed that the expression level of miR-451 (designated also as miR-451a) was considerably higher in endometriotic lesions compared to eutopic endometrium and in inverse correlation with macrophage migration inhibitory factor (*MIF*) level [11]. As miR-451 and *MIF* are shown to be primarily expressed in endometrial epithelial cells [47,74] then to ensure that the expression level differences between lesions and endometrium are not the consequence of different number of epithelial cells in tissues, the expression level of epithelial cell-specific cytokeratin 18 was determined and confirmed to be similar in both investigated tissues [11]. Therefore, the functional role of miR-451 in reducing expression of *MIF* was confirmed *in vitro* in endometrial epithelial cell line and the forced expression of miR-451 resulted not only in decreased *MIF* protein levels but also in reduced cell survival and proliferation. Authors proposed that the function of elevated miR-451 is to limit endometriotic lesion cell survival and on opposite, lesions expressing low levels of miR-451 have higher potential to survive [11]. Interestingly, *in vivo* induction of endometriosis in baboons caused significant reduction in endometrial miR-451 level that in turn led to the increase in its target gene *YWHAZ* expression [47]. Further, inverse correlation between miR-451 and *YWHAZ* expression was confirmed *in vitro* using epithelial cell lines and similarly to the previous study [11], overexpression of miR-451 inhibited cell proliferation [47]. However, in murine model of endometriosis, the low level of miR-451 was associated with a reduced ability of endometrial tissue to establish ectopically [75].

The higher expression of miR-202, previously associated with suppression of cell proliferation, migration and invasion in cancer studies [76], has also been reported in endometriotic lesions compared to endometria in whole tissue studies [16,18,20,48]. In a functional study, the high expression of miR-202 promoted cell proliferation and invasion through targeting transcription factor SOX6 and its downstream proteins [48].

The important role of miR-142-3p in the regulation of cell viability and pro-inflammatory signalling in EnSCs was shown using primary and immortalized EnSCs. This study confirmed that miR-142-3p upregulation causes significantly reduced expression of IL-6 co-receptor gp130 and suggested that miR-142-3p downregulation in endometriosis might lead to the activation of JAK/STAT signalling pathway and increased cell viability [77].

The role of angiogenesis in endometriosis pathogenesis is widely accepted, as neovascularization is needed for establishment of ectopic implants in peritoneal cavity. The dysregulation of some miRNAs, such as miR-15b, miR-16, miR-17-5p, miR-20a, miR-21, miR-125a, miR-221 and miR-222, that are shown to modulate the expression of pro-angiogenic vascular endothelial growth factor A (VEGF-A) and anti-angiogenic Thrombospondin 1 (TSP-1), has been shown in whole biopsy study [9]. Also, there was a significant inverse correlation between miR-424-5p expression and VEGF-A protein level in eutopic endometrium of women with endometriosis and an inverse correlation between miR-449b-3p and TSP-1 protein level in ovarian endometrioma [20]. To investigate whether VEGF-A expression is directly regulated by miR-16-5p, miR-29c-3p and miR-424-5p, as proposed by *in silico* analysis, primary stromal cells from endometrium of endometriosis patients and control women were transfected with respective miRNA mimics [78]. Interestingly, mimics transfection resulted in a significant down-regulation of VEGF-A protein level in a dose-dependent manner but did not affect VEGF-A mRNA expression, suggesting that these miRNAs mainly act as inhibitors of VEGF-A translation without

degrading respective mRNA. Furthermore, luciferase experiments demonstrated that *VEGF-A* 3' untranslated region (UTR) is a direct target of miR-29c-3p [78].

The relevance of other miRNAs in angiogenesis and disease pathogenesis has also been confirmed in functional studies. For example, miR-20a is an interesting anti-apoptotic miRNA that was shown to be downregulated [9,15,16] or upregulated [79] in endometriotic lesion biopsies. A functional study confirmed that miR-20a upregulation in EcSCs is transcriptionally induced by HIF-1 α and decreases the expression of its target protein DUSP2 [79]. This, in turn, leads to aberrant ERK activation, ERK-regulated gene overexpression and PGE2-induced *FGF9* expression. Because these genes play important roles in angiogenesis and stimulate both endothelial and endometrial cell proliferation, it was proposed that miR-20a plays an important role in endometriosis pathogenesis [79]. The results from the functional study suggested that also miR-199a may attenuate the angiogenic potential of stromal cells under the hypoxia partly through HIF-1 α /VEGF-A pathway suppression and thereby contribute to disease development [59]. Another study showed that elevated miR-199a in ectopic endometrial mesenchymal stem cells suppressed cell proliferation, motility and angiogenesis by targeting the 3' UTR of *VEGF-A* [80].

Interestingly, when considering miRNAs reported at least in three high-throughput studies, no overlapping miRNAs were found between all three study types and only miR-196b and miR-100 were common between low and high heterogeneity studies (Figure 2). A functional study in EcSCs proposed that overexpression of miR-196b causes downregulation of its target genes, a key regulator of cell proliferation *c-myc* and anti-apoptotic factor *Bcl-2*, leading to inhibition of endometrial cell proliferation and induction of apoptosis and mirroring the conditions in endometriotic lesions with low-level miR-196b and increased cellular proliferation [57]. Furthermore, there was a significant correlation between miR-196b and

HOXA10 expression and, thus, low miR-196b levels in endometriomas may stimulate proliferation, suppress apoptosis and induce lesion growth. The antiangiogenic miR-100 has shown to function as an endogenous repressor of the serine/threonine protein kinase mammalian target of rapamycin (mTOR) and modulate proliferation, tube formation, and sprouting activity of endothelial cells and migration of vascular smooth muscle cells [81]. The roles of miR-196b and miR-100 in cellular processes, such as proliferation and apoptosis, as well as in tissue vascularization and wound healing, makes them attractive candidates that can easily be associated with endometriosis pathogenesis.

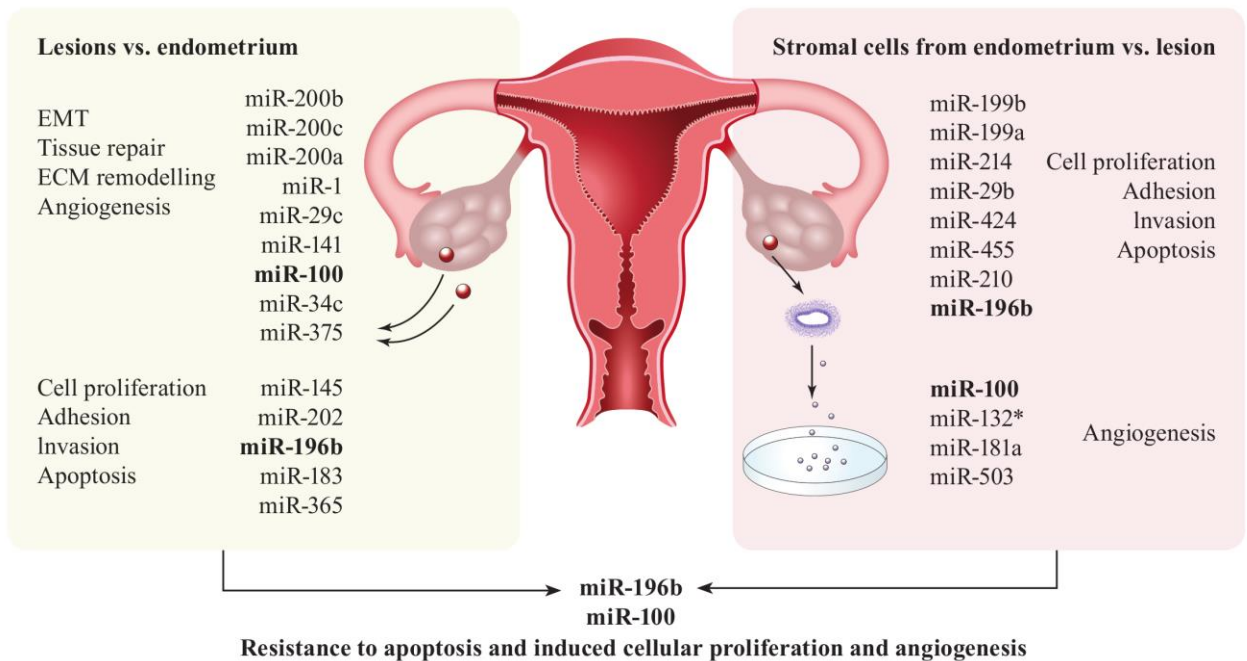


Figure 2. miRNAs and related biological processes reported in high-throughput miRNA studies in endometriosis. Figure denotes 14 miRNAs reported at least three times in high tissue/cellular heterogeneity studies (lesions compared to endometrium) and 12 miRNAs reported in a low tissue/cellular heterogeneity study (stromal cells from lesions compared to stromal cells from endometrium). ECM - extracellular matrix, EMT - epithelial-mesenchymal transition.

5.4 miRNAs regulating endometrial receptivity and infertility

Functional studies in endometriosis have also concentrated on miRNAs such as miR-135a/b and miR-199a, previously linked with menstrual cycle regulation and implantation failure, and thus potentially related to endometriosis-associated infertility.

The significant impact of menstrual cycle phase on miR-135a/b expression both in women with and without endometriosis was noticed by Petracco et al. [49]. It was shown that women with endometriosis have higher expression of miR-135a in proliferative phase endometrium, and higher miR-135b expression throughout the menstrual cycle compared to women without endometriosis. Furthermore, the authors showed that higher expression of miR-135a/b leads to downregulation of their target gene *HOXA10* and thereby suppresses endometrial receptivity in endometriosis [49]. That, in turn, may be the cause of endometriosis-associated infertility, as *HOXA10* has been proposed as one of the highly expressed genes in window of implantation [30]. Further, miR-199a has been linked with decreased endometrial receptivity and implantation defects in patients with endometriosis [45]. Transfection of endometrial cells with miR-199a repressed I κ B kinase/NF κ B signalling and inhibited IL-8 secretion, proposing that low expression of miR-199a upregulates inflammatory mediators leading to endometriosis-associated infertility [45].

Taken together, the knowledge obtained from functional studies widens our understanding of miRNA-mediated processes in endometriosis pathogenesis and may facilitate the development of potential therapeutics for endometriosis treatment. Ideally, the function of every dysregulated miRNA in endometriosis should be clarified before proposing it as a disease-related molecule. However, it should be borne in mind that miRNAs are a part of a complex cellular system, so that each miRNA regulates simultaneously several genes and, moreover, *in vivo* levels of miRNAs may be regulated by various internal environment

factors that are not embodied in *in vitro* conditions. Therefore, *in vitro* obtained results should be extrapolated with caution to complex biological systems, such as endometriotic lesions in peritoneal cavity.

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6. Future perspectives

Based on the above-discussed contradictory findings, we suggest that future miRNA studies should focus on the isolation and analysis of specific cells from lesions and endometrium (discussed in Section 2.1) rather than analysing the miRNA signature of whole lesion biopsies.

The large variability in tissue biopsy collection and characterization, specimen processing, and storage methodology, insufficient description of patient phenotypical data (e.g. missing data about menstrual cycle phase), small sizes of study groups and differences between miRNA detection methodologies, together with data analysis and interpretation differences, have created a situation where we still have no clear consensus on which miRNAs are involved in endometriosis pathogenesis. Thus, endometriosis studies need highly standardized sample collection recommendations, and large efforts to harmonize standard operating procedures for collecting both patients' phenotypical data and tissue samples have already been made. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project (WERF EPHeCt) has addressed the issues related to minimum and standard recommendations for data and sample collection emphasizing the importance of accompanying data registration during the tissue collection at laparoscopy [82–84]. This data should include menstrual cycle characteristics, data on hormone treatment, history of previous endometriosis surgery, as well as any imaging findings before the procedure, the type and duration of the procedure; the extent, exact location, and colour of endometriotic lesions, and also video/photo documentation of surgery [84]. Only harmonised data/sample collection between different research centres will open new possibilities to perform well-powered large-scale international collaboration studies and to increase the liability of the reported findings.

Not only issues related to sample collection and documentation are fundamentally important to uncover the role of miRNAs in endometriosis pathogenesis but also proper data analysis should be emphasized, as even small differences in data processing and analysis may have an impact on the study results. For example, in endometriosis candidate-miRNA quantitative-PCR studies the most commonly used internal reference gene for expression data normalization is U6 small nuclear RNA (RNU6B). Although being stable under physiological and pathological conditions and abundantly expressed in tissues, the suitability of U6 as reference gene in miRNA studies has been lately questioned [85,86]. Furthermore, as the expression of endogenous controls may be tissue-dependent, the best strategy is to establish stable endogenous controls in a particular experimental design. For example, non-coding RNAs (ncRNAs) RNU48, U75 and RNU44 were identified as stably and equivalently expressed between malignant and normal endometrial tissues while U6 was much less stable [87]. It may be argued that because of the larger size (approximately 200 bp), stability and processing efficiency of ncRNAs may differ from that of miRNAs; nevertheless, it has been shown that ncRNAs may perform as better endogenous controls than selected miRNAs [87]. Therefore, future studies should carefully consider the selection of best and optimal reference gene(s) as inappropriate normalization strategy may not only cause false association but leave small but significant changes in miRNA expression unnoticed [86].

7. Conclusions

The knowledge we have gathered from endometriosis miRNA studies suggests that the experimental design should be changed and should move from highly heterogeneous tissues to studies using specific cell populations. The methodology for obtaining pure fractions of cells from eutopic and ectopic endometrium without the need for cell culturing is already here (FACS, LCM) and constantly evolving, or as an alternative, computational methods as gene

expression profile deconvolution, can be used. Regardless of the approach chosen (experimental or computational), all different cell types (epithelial, stromal, immune cells) should be considered separately to reveal which specific cells are behind which particular aberrations, with the ultimate goal to uncover the molecular processes truly leading to formation of endometriotic lesions.

Conflict of interest statement

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Highlights

- Thus far, no consensus on which particular miRNAs are relevant in endometriosis
- Endometriotic lesions have higher cellular heterogeneity compared to endometrium
- Endometriosis-specific miRNAs could be masked by the tissue heterogeneity
- Cell-type specific miRNA profiles of endometrium and lesions should be determined