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Epigenetic Landscapes of Endometriosis: From Pathogenesis to Precision Medicine

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ABSTRACT

Endometriosis, a challenging gynecological disorder characterized by the ectopic presence of endometrial-like tissue, presents significant diagnostic and therapeutic hurdles due to its complex etiology and diverse clinical manifestations. Recent advancements in understanding its pathogenesis have underscored the pivotal role of epigenetic alterations, offering new insights into disease mechanisms and therapeutic targets. Epigenetic changes in endometrial cells significantly contribute to endometriosis pathogenesis, disrupting normal physiology and hormone responsiveness, particularly to progesterone. Dysregulation of histone modifications, DNA methylation, and non-coding RNA expression disrupts cellular homeostasis and promotes disease progression. Histone modifications, notably methylation and acetylation, influence chromatin structure and gene expression, affecting progesterone responsiveness and disease progression. Epigenetic regulators such as Cfp1 modulate progesterone receptor expression and downstream signalling pathways, presenting potential therapeutic targets. Non-coding RNAs, including miRNAs and lncRNAs, exert regulatory effects on gene expression and are implicated in endometriosis pathogenesis. Dysregulated expression disrupts cellular homeostasis and promotes disease progression. Biomarker studies have identified specific miRNAs and lncRNAs associated with endometriosis, offering avenues for non-invasive diagnosis and targeted therapies. siRNA-based therapies targeting key genes involved in endometriosis pathogenesis show promise as novel treatment modalities. By modulating gene expression and cellular functions, siRNA-based therapies offer a targeted approach to mitigate pathological processes. In this review, we summarize recent findings in the molecular mechanisms and regulatory pathways of endometriosis, offering valuable insights into pathology and therapeutic interventions. Future research efforts aimed at elucidating the complex interplay between epigenetic regulators and disease pathways hold promise for innovative diagnostic tools and targeted therapies.

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Introduction

Endometrium, the lining of the uterine cavity, is, undoubtedly, an extraordinary tissue. Its growth and maturation, which are extremely important for the embryo implantation, depend intimately on estrogen and progesterone concentrations. In case of the absence of conception, hormonal changes lead to the hypoxia and cell death in its functional (outer) layer, resulting in menstrual bleeding. Starting from the very first day of the menstrual cycle, the cells in the basal (inner) layer of endometrium start to mature and proliferate, in order to build a new functional layer in a few days, just in time for ovulation. This makes endometrium one of the fastest growing tissues in the human body (Maenhoudt *et al*., 2022).

Another striking characteristic of endometrium is the fact that in some patients it can be found outside its natural location. This benign pathological condition, called endometriosis, is defined as a presence of functional endometrium outside the uterus (MeSH database). Most often, the endometriotic foci, or ectopic endometrium, are found in the peritoneal cavity (Saunders and Horne, 2021) but can be found in more distant organs as well (Andres *et al*., 2020). Just like the eutopic endometrium, the ectopic one goes through a hormone-dependent cycle of growth and bleeding. Additionally, processes such as inflammation (Machairiotis *et al*., 2021), epithelial -to-mesenchymal transition (EMT) (Proestling *et al*., 2015), angio- (Rocha *et al*., 2013) and neurogenesis (Asante and Taylor, 2011) are observed in endometriosis. Several cancer-driving mutations have been discovered in the ectopic lesions of patients with this condition (Anglesio *et al*., 2017), although it's important to mention, the observed changes might be a result of the endometriosis progression rather than a reason for its development (Guo, 2018).

Endometriosis affects about 10% of women worldwide and is associated with pain (Bellelis *et al*., 2010), infertility (Filip *et al*., 2020) and increased predisposition to cancer (Pearce *et al*. 2012; Kok *et al*., 2015). Despite this disease being a social-economic burden (Missmer *et al*., 2021; Darbà and Marsà, 2022), mechanisms responsible for its pathogenesis are still unknown; this slows down both the process of discovering new, more effective treatments that do not interfere with patients' ability to conceive and the non-invasive yet reliable diagnostic tests.

The theory of implantation through retrograde menstruation proposed almost a century ago till date remains the most well-known and the best-supported in the scientific community (Sampson, 1927). It suggests that during menstruation some amount of endometrium cells can travel with blood through fallopian tubes to the peritoneal cavity, then attach and form the endometriotic lesions. This theory, however, ignores cases of endometriosis in men (Martin and Hauck 1985; Schrodt *et al*., 1980), same as the presence of ectopic endometrium in species that don't menstruate, such as guinea pigs (Baldi *et al*., 2017). It also does not necessarily explain why only a portion of women experiencing retrograde menstruation develops endometriosis (Halme *et al*., 1984).

Epithelial to mesenchymal transition of endometrium cells can be another way to explain the onset of the disease. During this process EMT transcription factors, such as zinc finger E-box-binding proteins (Zeb1/Zeb2), repress the expression of epithelial cell junction elements, for example E-cadherin, encoded by

Cadherin-1 (*CDH1)* gene (Du *et al*., 2019). Furthermore, TET1 activation leads to simultaneous *CDH1* downregulation and enhanced synthesis of N-cadherin. Obtained mesenchymal phenotype of cells allows for migration and invasion of other organs (Wu *et al*., 2020).

For the progression of endometriosis, the endometrial implant must be protected from apoptosis and immune response. Upregulated expression of cyclooxygenase-2 (COX-2) promotes the production of prostaglandins, consequently leading to immune evasion (Wang *et al*., 2012). Increased prostaglandins' levels promote angiogenesis via stimulating vascular endothelial growth factor (VEGF) overexpression, which increases the supplies of nutrients (Tamura *et al*., 2006). That, alongside the increased estrogen concentration, leads to endometrial tissue growth stimulation. The estrogen action on ectopic endometrium is regulated by progesterone, which by binding to its receptor induces downregulation of the estrogen receptors. However, the loss of progesterone receptors allows for endometrial cell proliferation, independent of progesterone (Reis *et al*., 2020). Furthermore, obtained ability to either synthesize estrogen, acquired via steroidogenic factor-1 (SF-1) activation, or convert extracellular testosterone to estrogen, due to aromatase production, accelerates the growth of endometriotic tissue (Xue *et al*., 2007b).

The overexpression of *Insulin-like growth factor 1 receptor (IGF1R)* in ectopic endometrium positively affects both endometrium cells migration and proliferation (Bai *et al*., 2021). This effect is enhanced by decreased levels of *Homeobox A10 (Hoxa10)* expression product, which allows for epithelial- to mesenchymal transition and estrogen synthesis (Elias *et al*., 2023) (Fig. 1).

Endometriosis is considered a complex disease, where there is no specific gene responsible for its development, but a set of genomic and environmental alterations. Given that, data from Genome Wide Association Study (GWAS) can serve as a valuable source of information for possible etiopathology (Cano-Gamez and Trynka, 2022). As some of the single nucleotide polymorphisms identified through GWAS are located in independent non-coding DNA fragments involved in gene expression regulation, it was suggested that epigenetic modifications contribute to endometriosis development (Zondervan *et al*., 2016).

Epigenetics studies gene expression changes without interfering in DNA sequence. Those changes are possible because of the epigenetic modifications. They can activate or inhibit gene transcription by chromatin modifications. Genetic modifications involve several mechanisms. There are DNA methylation, histone modification and non-coding RNA. DNA methylation interferes with gene expressions connected with implantation. Histone modifications are important in pathogenesis in endometriosis. It suggests that inhibitors of histone modifications enzymes may have their role in endometriosis treatment. In endometriosis there are over 50 different expressions of miRNA. MiRNA is type of non-coding RNA (Adamczyk *et al*., 2022). Such changes can be passed on to offspring and might explain a partially hereditary nature of endometriosis (Simpson *et al*., 1980). Role of epigenetics in endometriosis is mainly referring to development of endometriosis (Adamczyk *et al*., 2022).

In recent years, numerous studies on epigenetic regulation of endometriosis have been published, revealing the striking differences in epigenetic

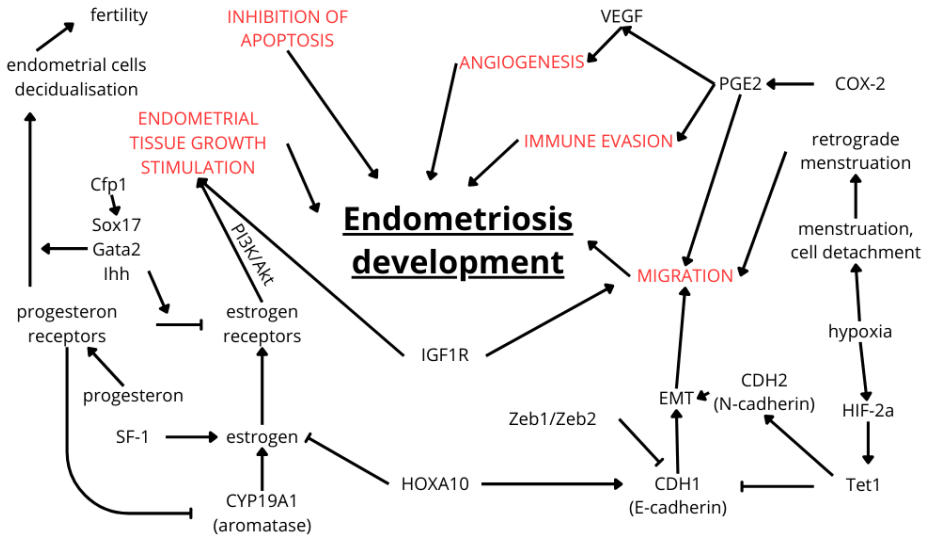

Figure 1. Processes leading to endometriosis development. Endometriosis is characterized by several processes which comprise the pathogenic phenotype. These include migration, endometrial tissue growth stimulation, inhibition of apoptosis, angiogenesis and immune evasion (enumerated and described respectively in the following text). I) Migration is fostered by the following factors. Physiological hypoxia during menstruation, which allows for cell detachment, upregulates *Tet1* through *HIF-2a* transcription activation. This promotes EMT through inhibition of *CDH1* (encoding E-cadherin) and activation of *CDH2* (encoding N-cadherin) expression. HOXA10 promotes *CDH1* expression. Conversely, Zeb1 and Zeb2 downregulate it. Migration can be promoted by IGF1R and also by PGE2 action, the level of which is elevated after COX-2 overexpression. II) Endometrial tissue growth is regulated by estrogen receptor activation (ER) followed by PI3K/Akt activation. ER activation is inhibited by progesterone receptor (PR) action, which is promoted by Cfp1. PR downstream pathway inhibits aromatase function, which is to convert androstendione to estrogen. The latter can be produced intracellularly after SF-1 expression activation in endometrial cells. HOXA10 inhibits the aforementioned process. IGF1R promotes the growth of endometrial tissue. III) Inhibition of apoptosis that can be introduced by downregulation of suppressor genes like *TP53* (not included on the figure). IV) Angiogenesis is induced by VEGF overexpression that can be caused by PGE2 action. V) Immune evasion also is promoted by PGE2. Additionally, Cfp1 provides proper decidualization by acting on PR downstream pathway, what is crucial for maintaining fertility.

regulation of patients with endometriosis and healthy women. In this review, we discuss and streamline some of that work, to illuminate possible molecular mechanisms underlying causes of endometriosis and discuss potential treatment and diagnostic opportunities.

Deacetylation and methylation of histones may have a critical role in endometriosis progression

Epigenetics plays a key role in regulating gene expression without simultaneously affecting the DNA sequence. One of the well-studied mechanisms included in epigenetics is post-translational modifications of histones (Weinhold, 2006). On their N-terminal and C-terminal tails are amino acid residues such as lysine, arginine, serine or threonine (Alaskhar Alhamwe *et al*., 2018). These amino acid residues undergo acetylation, methylation and phosphorylation as modification processes of histones (Zhang *et al*., 2021).

The mechanisms of histone acetylation and deacetylation mainly occur via two enzymes: histone acetyltransferases

(HATs) and histone deacetylases (HDACs). The role of the mechanism of histone acetylation is not known yet. However, the role of the deacetylation mechanism and its occurrence in pathological endometriosis tissue has been demonstrated. Decreased level of histone 3 (H3) acetylation has been found in the endometrial lesions; in particular, the decreases level of acetylation of histone 3 on lysine 9 (H3K9) is mainly indicated here. H3K9 has influence on expression of *p16*, *MLH1* and *HOX* genes. High expression of *HOXA10* has influence of endometriosis progression. Hypoacetylation of H3 causes lower transcription of *HOXA10*. These genes are related to the endometriosis progression (Monteiro *et al*., 2014). However, despite the general lack of differences in histone 4 (H4), it is worth noting that the same study also presented clear evidence of decreased level of acetylation of histone 4 on lysine 16 (H4K16) in patients diagnosed with endometriosis. Deacetylation of the above histone lysine residues highlights the role of HDACs in the development of endometriosis (Monteiro *et al*., 2014) and inhibition of HDACs that causes a reduction of invasion and proliferation on endometrial cells in an animal model (Wu *et al*., 2007).

The study to understand the changes in HDAC expression levels in endometriosis started with histone deacetylase 1 (HDAC1) and histone deacetylase 2 (HDAC2). The HDAC1 expression was higher in endometriosis samples compared to control samples, however, the origin of the tissue showing the changes present in endometriosis was important (Colón-Diaz *et al*., 2012). The HDAC2 has also been identified as a factor that affects the development of endometriosis, as it has been shown to be highly expressed in the endometriosis cells. Furthermore, silencing the gene of HDAC2 induced apoptosis for cells and inhibition of endometriosis progression. Activation of the HNFA4/ARID1A axis has been found in endometriosis progression (Fig. 2). HNF4A is a

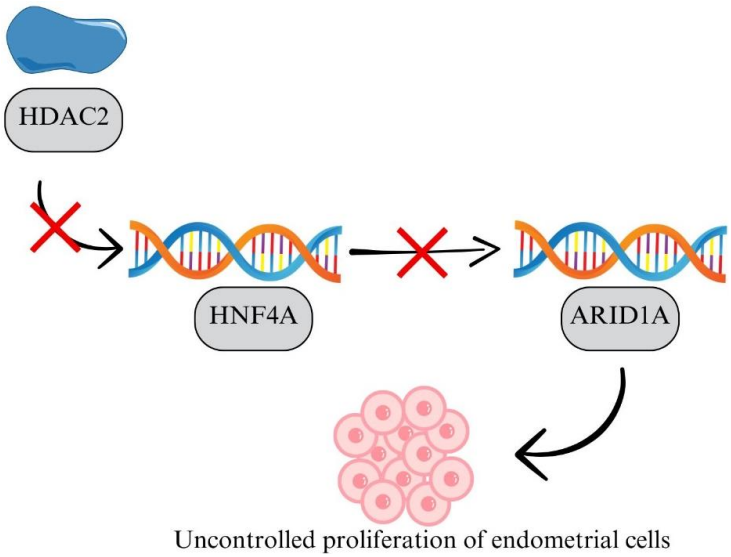

Figure 2. High level of histone deacetylase 2 (HDAC2) may lead to progression of endometriosis by inhibition of HNF4A/ARID1A pathway.

transcriptional factor promoting the expression of other genes such as *ARID1A* and others that affect endometrial cells progression. The study shows that higher level of HDAC2 effects on silencing of *HNFA4*. That means, the lower level of *HNFA4* may silence the expression of *ARID1A*. That means uncontrolled proliferation of endometrial cells and progression of endometriosis (Mai *et al*., 2021).

In the HDAC family there is also a Sirtuin 1, whose elevated expression has been correlated with endometriosis and formation of inflamed tissue, particularly with the ovarian endometriosis type (Taguchi *et al*., 2016; Mvunta *et al*., 2017). On the other hand, abnormal expressions of HDAC1 and histone deacetylase 6 (HDAC6) were clearly noted in deeply infiltrating endometriosis (Zheng *et al*., 2023). At the same time, attention was drawn to histone deacetylase 8 (HDAC8), whose inhibition of expression in deeply infiltrating endometriosis by using an HDACs inhibitor reduced lesion development and endometriosis progression by two-thirds (Zheng *et al*., 2023). HDAC and its role of endometriosis progression is not well studied. They seem to be very successful therapeutic targets. However, their influence on certain genes needs to be found. Still there are not many studies about this topic, which is important for better understanding of endometriosis progression.

Methylation also occurs predominantly on the lysine residues of histones, mainly H3 and H4. It is mainly catalysed by histone methyltransferase (HMT). This leads to chromatin compression and regulation of gene expression (Zhang *et al*., 2021). However, there is a mechanism of histone demethylation carried out by the enzyme histone demethylase 1 (LSD1) (Li *et al*., 2024). H3K9 and H3K4 demethylation are linked with LSD1. LSD1 influences on *CDH1* causing its downregulation of expression. It means that there is a high chance of starting EMT in endometriosis (Ding *et al*., 2014).

Higher levels of histone methylation were observed in endometriosis than in normal tissue (Monteiro *et al*., 2014). Level of methylation of histone 3 on lysine 4 (H3K4) is a much higher in the endometriotic than in the control samples, whereas an increase in methylation of H3K9 was also noticed in the same samples but comparably not as high. H3K9 has influence on *HOXA10* gene expression. Hypermethylated *HOXA10* is involved in endometriosis progression. By far the most interesting result was obtained for the levels of methylation of histone 3 on lysine 27 (H3K27), which was found in both eutopic and ectopic lesions in patients diagnosed with endometriosis (Monteiro *et al*., 2014). Further investigations showed that the highest expression in endometriosis was from 3-methylated lysine 27 of histone 3 (H3K27me3). This methylation model is catalysed by the enhancer homologue of zeste 2 (EZH2), whereas increased expression in endometriosis (Colón-Caraballo *et al*. 2015). Furthermore, many H3K27me3 are in the promoter regions of candidate genes as biomarkers in endometriosis, including genes of estrogen receptor 1 (*ESR1*), cadherine 1 (*CDH1*), and progesterone receptor (*PGR*). Especially there is focus on the *CDH1* and the CDH1 methylation in this case (Colón-Caraballo *et al*., 2018). *CDH1* is hypermethylated in endometriosis lesions, which means this gene's expression is lower. Downregulation of *CDH1* promotes the development of EMT (Monteiro *et al*. 2014). There is still too little information about histone methylation of *ESR1* and *PGR* and its influence on development of endometriosis.

All these facts render the HDAC inhibitor a new therapeutic target, whereas an understanding of the effect of HDACs in the pathogenesis of endometriosis is required. However, it is important to remember that epigenetic aberrations basis of endometriosis is highly dependent on the development of endometriosis (Zheng *et al*. 2023, Colón-Diaz *et al*. 2012). H3K27me3 has also increased in endometriosis. That makes H3K27me3 the major epigenetic marker for endometriosis. Furthermore, inhibition of EZH2 is considered as a target in the treatment of endometriosis (Colón-Caraballo *et al*., 2015).

Women with endometriosis have an altered DNA methylation profile

The most abundant DNA modification is methylation of cytosine to 5C-methylcytosine (5-mC). Other oxidated forms of 5-mC (5-hydroxy-methylcytosine [5-hmC], 5-formyl-cytosine [5-fC], 5-carboxylcytosine [5-caC]) and methylation of adenine to N6-methyladenine (6-mA) are less common and therefore less examined, yet their role in transcription regulation is pivotal (Klungland and Robertson, 2017). Cytosine modification takes place mainly within regions of high cytosine (C) and guanine (G) density sequences named CpG islands.

CpG islands have been found in about 70% of human gene promoters and their role in transcription regulation has been proven robustly (Al Aboud *et al*., 2023). Gene transcription can be both silenced by CpG 5mC methylation within promoters and, less commonly, silenced or enhanced by CpG methylation in other functional parts of genes. These changes can be recovered thanks to DNA active demethylation. The process of methylation is guided by DNA methylotransferases (DNMTs) and the demethylation is conducted by ROS1 and also by Tet and TDG pathways (He *et al*. 2011; Onodera *et al*., 2021). The 5-hmC, 5-fC, and 5-caC serve as intermediate states preceding demethylation but independent biological functions of 5-hmC and 5-fC are also suspected (Kumar *et al*., 2018). While methylation of gene promoters and first exon in gene body is percieved as transcription downregulating factor, methylation within rest of gene body can be actually an enhancer of its transcripton in in dividing cells (Moore *et al*., 2013) Taking into consideration that endometrial and endometriotic cells proliferate in high rate, this dependence may apply to them.

TET methylcytosine dioxygenase genes were claimed to be downregulated in endometrium of women with endometriosis and endometriotic lesions compared to control samples. However, at the same time 5-hmC content in tissue was increased, whereas decreased in blood (Roca *et al*., 2016). This wasn't confirmed in other study (Yotova *et al*., 2017), therefore aforementioned contradictory results could have arisen from some mistake. Another study indicates that in general Tet family expression is dysregulated in endometriotic cells (Wu *et al*., 2020). Representants of TET family are expected to exhibit specificity in their targets of demethylation, therefore it is important to examine the gene expression individually for its members. The study concentrated on methylcytosine dioxygenase TET1, encoded by *TET1*, function and expression in endometriotic tissue. Protein encoded by this gene has been shown to upregulate N-cadherin (mesenchymal adhesive protein) while downregulating E-cadherin (epitelial adhesive protein) in epitelium of eutopic endometriotic cells. This trait points to TET1 protein as a crucial factor of EMT. Authors present results indicating upregulation of *TET1* expression in epitelium of eutopic endometriotic cells.

It is suggested that hypoxia induced *HIF-2α* (Hypoxia induced factor 2α) transcription factor expression upregulates *TET1* gene. Hypoxic conditions arise while endometrial epitelial cells detach during menstruation. Then, during retrograde menstruation, they change adhesion profile from epithelial to mesenchymal, which allows for cell survival while not attached to basal layer. E-cadherin is a part of pathway that leads to anoikis during disconnection from the layer (Kumar *et al*., 2011). Substitution of this adhesive protein with N-cadherin helps to avoid anoikis during cell migration.

Numerous studies present genes that show different methylation patterns between control and endometriosis samples. These include: hypomethylation of coding region in gene coding the aromatase (*CYP19A1*) what leads to upregulation of gene expression (Izawa *et al*., 2011); homeobox A10 (*HOXA10*) promoter hypermethylation (Elias *et al*. 2023); estrogen receptor 2 (*ESR2*) (Xue *et al*. 2007a), steroidogenic factor-1 (*SF-1*) (Xue *et al*., 2007a) and cyclooxygenase-2 (*COX-2*) (Wang *et al*., 2012) promoter hypomethylation.

Aromatase is an androstendione to estradiol converting enzyme. As the most potent from all estrogens, estradiol promotes proliferation of cells expressing estrogen receptor, including endometriotic ones. Overexpression of this enzyme's gene in ectopic stromal cells allows for more aromatase production and higher levels of estradiol inside the cells, leading to increased cell division and implant growth.

Homeobox protein Hox-A10 (homeobox A10) encoded by *HOXA10* is a DNA-binding transcription factor which is responsible for controlling the expression of genes in stromal cells of endometrial tissue during secretory phase, when differentiation from fibroblast-like to decidual cells takes place. The hypermethylation of *HOXA10* promoter has been discovered in eutopic but not ectopic endometriotic cells. This may indicate that silencing *HOXA10*, which results in undifferentiated cellular development state, is essential for cell detachment and migration, though hypermethylation has to be retreated to let for ectopic implantation. It is consistent with homeobox A10's function as a repressor of Snail gene (*SNAI1*), product of which is a repressor of E-cadherin (Yoshida *et al*., 2006). *HOXA10* has been also connected with downregulation of enzymes for cholesterol synthesis, which is a substrate in estrogen production (Yu *et al*., 2022). Therefore, *HOXA10* silencing may increase estrogen production in endometriotic cells.

Estrogen receptors are required for both endometrial and endometriotic cells to proliferate. Functional particles are dimers formed by subunits α and β as homo or heterodimers. ERβ, encoded by ESR2 gene, expression is upregulated in ectopic tissue cells compared to eutopic ones. This is not the case with ERα encoded by ESR1 gene on the other hand. Methylation of CpG islands within promoters of these genes reflect the mRNA expression level. This result is intriguing, as ERβ homodimers show anti-proliferating activity after binding the estrogen in a murine cellular model (Song *et al*., 2022). ERα is the estrogen receptor subunit homologue, which is overexpressed in cancers like breast cancers, and it exhibits a proliferation stimulating trait. There is a possibility of the model not being translative to human cells *in vivo*, but currently the hypomethylation of ERβ does not seem to be beneficial to endometriotic cells but the real gain may yet to be discovered.

Steroidogenic factor-1 is a transcription factor responsible for expression of machinery for estrogen

biosynthesis. Even though the process is one of physiological sources of estrogen, it does not happen in endometrial stromal cells due to SF-1 promoter hypermethylation. Hypomethylation of CpG island within this locus allows for endometriotic cells for self-synthesis of estrogen, which through estrogen receptors promote the proliferation of the cell.

Cyclooxygenase-2 takes part in enzymatic conversion of arachidonic acid to prostaglandins. CpG island within promoter of the gene it is encoded by in normal endometrium is hypermethylated. Expression of *COX-2* leads to inflammation through prostaglandins production. Further activation of molecular pathways promotes cell survival, invasion, angiogenesis and immune evasion. These traits allow endometriotic cells to migrate beyond uterus, when *COX-2* expression is elevated in case of their promoter hypomethylation. PGE2 also mediate the angiogenesis through *VEGF* overexpression, leading to enhanced nutrition of intensively proliferating tissue of endometrial implants (Tamura *et al*., 2006).

As shown, many genes, particularly those connected with proliferation, cell signalling, migration, immunity, angiogenesis, undergo changed methylation content in endometriosis compared to control (Mortlock *et al*., 2023). This stresses the importance of methylation level in endometriosis pathomechanism. In the epithelial cells of endometrium of women with endometriosis, 5-hmC contents are lower than in control, although parallel to 5-mC, indicating general trend of demethylation (Yotova *et al*., 2017).

Although not commonly seen in DNA, N6-methyladenine (6-mA) has been classified as an RNA modification. Shen *et al*. proved also the influence that 6-mA can have on endometriosis development. Functionality of methyltransferases conducting methylation of adenosine is crucial for post transcriptional use of mRNA. It has been proven that 6-mA content in mRNAs coding proteins from PI3K/Akt pathway affects translation performance. The pathway is responsible for cell survival, proliferation, estrogen signalling. Therefore, enhanced translation caused by hypermethylation of adenosine in these transcripts can promote endometriotic lesion growth (Shen *et al*., 2023).

DNA methylation is a double-edged sword when it comes to functional traits – it can silence transcription of genes both beneficial and unfavorable for disease development. Therefore, to utterly understand the molecular basis behind probable impacts of DNA modifications on the mechanisms of selectivity of this phenomenon need to be uncovered.

Enhancer methylation has been proven more correlated with gene expression than promoters' methylation in breast cancer (Aran and Hellman, 2013). This study stresses the importance of enhancer sequence methylation. It is therefore crucial not to confine research to mere promoters, but to also study the role of enhancers and the coding regions also in endometriosis pathology.

Some studies focus on discovering the epigenetic differences between samples of eutopic and ectopic foci (Mortlock *et al*., 2023), which at first glance is the proper way to evaluate the shift which the endometrial cell must make to migrate to ectopic loci. Unfortunately, the rate of specific cell types differs in constitution of eutopic endometrium compared to ectopic lesion. Therefore, while analysing any markers with purpose of comparison between these two sources, this inequality has to be taken into consideration (Barjaste *et al*., 2019).

Etiopathological changes may well be present already in the cells of endometrium (Li *et al*., 2019). Changes in expression of genes from pathways which play a key role in endometriosis can be found throughout the surface of patients' uteri (Brosens *et al*., 2012). This phenomenon has a twofold consequence. Firstly, it allows us to diagnose the disease early and in a non-invasive way, thanks to biomarkers detected in aspiration biopsy material from eutopic endometrial tissue (Żeberkiewicz *et al*., 2022). Secondly, it sheds more light on the character of endometriosis: when pathogenic changes are present in the progenitor cells throughout uterus, a foundation of the disease has been inherited or developed up to the moment of organogenesis. Then the pathogenic traits get activated at the onset of puberty with the change in sex hormones release. Alternatively, the pathogenic traits were gained due to environmental factors which have driven epigenetic change in most endometrial cells or cells that control their functioning. Even taking into consideration a delayed diagnosis (Nnoaham *et al*., 2011), substantial part of cases onset after the age of 30 (Gunawardena *et al*., 2020). Therefore, it is possible that the inherited mutations but also the modifications gained through individual's life can modulate the pathogenic phenotype (Afshar *et al*., 2013). This points to epigenetics as the probable driver of these changes (Martin and Fry, 2018).

Epigenetic changes are responsible for progesterone resistance in endometriosis pathogenesis

Understanding of the epigenetic changes such as methylation or acetylation of DNA that underlie endometriosis requires comprehension of the dynamic physiology of endometrium, which is widely regulated by female sex hormones such as progesterone and estrogen. The first one – progesterone (P4) is very important both for monthly development of endometrium and during pregnancy, and especially for the embryo implementation into the uterine wall. The molecular mechanisms of P4-driven regulations depend on activation of its receptors (progesterone receptors; [PGRs]). The PGRs control expression of local factors such as those responsible for epithelium-stroma interactions which play a clue role in endometriosis pathogenesis. The well-known methylation and acetylation patterns of H3/H4 in women with endometriotic lesions are highly distinct in endometrium of the healthy patients. These changes can interrupt the physiological response to progesterone and estrogen (Yang *et al*., 2023).

One of the genes indirectly affecting the uterus response to progesterone is *Cfp1*, which encodes the zinc finger protein 1. Due to its numerous binding domains, *Cfp1* induces epigenetic changes in two ways: At the DNA level by interfering with DNA methyl transferase 1 (DNMT1) activity, and at the histone level by attaching the SET1 subunit to the H3K4 methyltransferases, which enables its trimethylation. The CXCC domain binds DNA at the unmethylated CpG islands; this prevents the formation of inappropriate H3K4 methylation patterns outside these regions and thus maintains the appropriate epigenetic structure of chromatin (Fan *et al*., 2023). *Cfp1* is an evolutionarily conserved gene and its silencing in the embryonic cells is closely related to a decrease in cytosine methylation, by decreasing the translation efficiency and the methyltransferase stability. In addition, *Cfp1* affects the levels of expression of *Gata2*, *Sox17* or *Ihh* genes involved in the signalling pathways of cellular response to progesterone (Yang *et al*., 2023).

The loss of the *Cfp1* gene and ergo lack of that gene's translated protein was

correlated with mouse infertility, which was caused by several disorders from embryo transport to subsequent implantation, including disorders of endometrial cell proliferation (Yang *et al*., 2023). Additionally, abnormalities in the inhibition of ectopic endometrial growth by P4 were observed in a mouse model of endometriosis with *Cfp1* deletion. The deletion is associated with a significant decrease in expression of *Gata2, Sox17* and *Ihh* genes (Yang *et al*., 2023). These observations became the basis for the study of Cfp1-dependent progesterone resistance in human endometrial foci cells. For this purpose, transcripts of healthy patients and patients with endometriosis were compared (Yang *et al*., 2023). Despite the lack of association between the level of *Cfp1* expression and the levels of expression of the progesterone receptor gene, a similar decline in expression of the *Gata2, Sox17* and *Ihh* genes was observed in the cells of women with endometriosis, similarly to the mouse model (Yang *et al*., 2023). However, noticeable differences in the expression of these genes were found only in women with severe disease (Yang *et al*., 2023). These findings indicated the role of *Cfp1* in the pathogenesis of endometriosis and helped formulate the hypothesis of disruption of signalling pathways in the cellular responses to progesterone in cells following the epigenetic changes (Yang *et al*., 2023).

Altered non-coding RNA expression profile can lead to endometriotic lesions

The term 'non-coding RNA' (ncRNA) refers to the RNA fragments that do not encode for a protein, but are involved in other important processes, to which the post-transcriptional gene expression regulation belongs (Hombach and Kretz, 2016) Some ncRNA, such as microRNA (miRNA), small interfering RNA (siRNA), and long non-coding RNA (lncRNA or lnRNA) have been identified as potential key players in endometriosis development (Abbaszadeh *et al*., 2023).

MicroRNA are short RNA sequences, predominantly 22 nucleotides in length; when bound to mRNA, microRNAs serve as a signal for its degradation by ribonucleases. In that way miRNA is able to influence the expression level of targeted genes (Hombach and Kretz, 2016).

The 2020 case-control study showed the difference in the miR-125b levels between endometrium (eutopic or ectopic) in patients with endometriosis and the healthy controls. It was proposed that the increased activity of miR-125b interferes with TP53 expression, resulting in inhibition of apoptosis (Hajimaqsoudi *et al*., 2020).

A microRNA profile analysis of patients' ectopic endometrial tissue using new generation sequencing (NGS) (Hawkins *et al*., 2011) resulted in 10 upregulated (miR-202, 193a-3p, 29c, 708, 509-3-5p, 574-3p, 193a-5p, 485-3p, 100, 720) and 12 downregulated (miR-504, 141, 429, 203, 10a, 200b, 873, 200c, 200a, 449b, 375, 34c-5p) miRNAs when compared to healthy endometrium of the controls.

Interestingly, a different study confirmed an increase in expression of miR-29c, this time in eutopic endometrium of endometriosis patients; miR-29c involvement in endometrial tissue progesterone resistance through downregulation of its targeted gene's level (FK506-binding protein 4) was shown as well (Joshi *et al*., 2017). Additionally, both the miR-200 family and the miR-34c-5p have been associated with epithelial-to-mesenchymal transition. miR-200s inhibit Zeb1 and Zeb2 transcription factors, as well as MALAT lncRNA (Du *et al*., 2019), while miR-34c-5p inhibits the Notch1 signaling. Downregulation of those microRNAs

results in the loss of epithelial phenotype and increases cells' migration and invasion abilities in the in vitro models of endometriosis (Luo *et al*., 2020).

Several studies have focused on serum miRNA profiling with the aim of creating a new non-invasive biomarker for endometriosis (Cosar *et al*., 2016; Chico-Sordo *et al*., 2024). For example, serum levels of circulating miR-31 and miR-145 turned out to be significantly different in women with and without endometriosis (Bashti *et al*., 2018).

Combining microRNA research and artificial intelligence allowed to differentiate between patients with endometriosis and controls with exceptional accuracy and test's AUC (area under the curve) value reaching up to 98.4% (Bendifallah *et al*., 2022).

Long non-coding RNAs define the RNA sequences exceeding 200 nt in length which do not undergo translation. But, lncRNAs undergo splicing, polyadenylation, and capping, similar to mRNA (Guttman *et al*., 2009). The lncRNA are able to act on gene expression by employing four main molecular mechanisms, including of modifications protein: protein interactions, small RNA binding, miRNA inhibition through competition, and guiding RNAs to RNA-binding proteins (Statello *et al*., 2021). The way lnRNAs can serve as an epigenetic agent is therefore through interference with functioning of small RNAs and proteins that control gene expression. Recently, lnRNA was proven to regulate chromatin structure, thus influencing transcription process (Yan and Bu, 2021).

Based on bioinformatics analysis, 3 lnRNAs were found as strongly associated with endometriosis development, allowing for functional explanations of pathological traits they supply (Bai *et al*., 2021). One of them, H19, inhibits miRNA let-7 leading to IGF1R upregulation and, in consequence, to enhanced proliferation and migration of endometrial stromal cells (Bai *et al*., 2021). Another one, GS1-358P8.4, interrupts the Rap1 signalling pathway, thus providing cancerous traits. The last one – RP11-96D1.10, comparably the least studied, seems to supply a trait common with H19.

In another study, SNHG4 lnRNA that promotes proliferation, migration, epithelial – mesenchymal transition, and suppresses apoptosis, has been proven to be overexpressed in uteri of women with endometriosis, suggesting possibility of implementing this lnRNA in diagnostic assays (Szaflik *et al*., 2023).

Small interfering RNA (siRNA) plays a pivotal role in defence against RNA viruses. Its ability to complementarily guide the RNA degradation by utilizing RISC allows it to overpower the invader but also control the copy number of transcripts (mRNA) (Zhang, 2023). This feature can be applied to treat diseases that result from overexpression of specific genes. Therefore, such treatments can find a potential use in endometriosis therapy. For example, vascular endothelial growth factor (VEGF) and ribonucleotide reductase subunit M2 (RRM2) have been proposed as therapeutic targets as proof-of-concept for novel treatments for endometriosis (Kiisholts *et al*., 2021). The VEGF provides the cell with the ability to induce angiogenesis in its environment, whereas the RRM2 promotes proliferation and epithelial-mesenchymal transition, among other processes (Kiisholts *et al*., 2021). Constraining the concentration of their mRNA in endometriotic cells cytoplasm restrains overproduction of proteins; this in turn endows the pathogenic cells with traits useful for migration to and survival in ectopic locus (Kiisholts *et al*., 2021).

Therapeutic and diagnostic perspectives for targetting the epigenetic changes in endometriosis

Discovering the epigenetic mechanisms underlying endometriosis provides us not only with the knowledge itself. Awareness of specific molecules and pathways involved in pathogenesis can be utilized as treatment targets and diagnostic markers. Among the former there are HDAC family and EZH2. The silencing of these genes and inhibition of proteins they encode have shown a potentially therapeutic effect, also on animal models. Another possibility of treatment had an advent along with exploration of siRNA translation downregulating effect on mRNA coding proteins that promote endometriosis development. Implementation of siRNA which silent expression of genes, for instance VEGF and RRM2, can revolutionize the state of the art in endometriosis treatment.

Lack of effective treatment of endometriosis is not the only problem that remains yet unsolved. Non-invasive tests for endometriosis diagnosis are still not implemented as standard procedure on behalf of invasive laparoscopy and USG which lacks sensitivity (Allaire *et al*., 2023). Minimally invasive tests employing biomarkers acquire increasingly relevant performance. DNA modification is another epigenetic mechanism which impacts gene transcription efficiency. Basing on differences in specific mRNA levels in tissue samples of endometrium, a diagnostic test has already been developed (Żeberkiewicz *et al*., 2022). There are promising results of studies which put histone methylation under examination. `3-methylated lysine 27 of histone 3 is significantly more abundant in patients compared to control samples. This fact places it among potential endometriosis markers.

Non-coding RNAs, particularly microRNAs and long non-coding RNAs, have emerged as key regulators of gene expression and potential non-invasive biomarkers for disease diagnosis and prognostication, and novel therapeutic targets for intervention. Tests that measure the concentration of lnRNAs and miRNA prove their utility while showing significant performance.

Conclusions

For over a century endometriosis's etiopathology remains an unsolved mystery. However, recent advancement in molecular biology techniques provided researchers with tools to investigate the disease's underlying causes. There is a growing body of evidence that epigenetic modifications might play a crucial role in endometriosis development. Studies have shown that patients with endometriosis resemble significantly different patterns in histone modifications, DNA methylation and ncRNA expression. Epigenetic alterations emerge as critical determinants in endometriosis, influencing hormone responsiveness, gene expression profiles, and disease progression.

Histone acetylation patterns, mainly of H3K9, introduced by HATs and rescued by HDACs differentiate normal from endometriosis's epigenetic landscape. HDAC members level in endometriosis are elevated leading to survival (HDAC2), inflammation (Sirtuin 1) and infiltration of peritoneal layer (HDAC1, HDAC6 and HDAC8).

Methylation level of H3K27me3 in histones associated with promoters of genes important in endometriosis development was elevated in ectopic endometrium in patients with endometriosis and control endometrium in secretory phase compared to control in proliferative phase. The same dependency was claimed in the case of EZH2 protein

level, which catalyses this type of methylation (Colón-Caraballo *et al*., 2015). It is claimed that H3K27me3 repressing mark was put in regions of promoters of *PGR* and *ESR1* (Colón-Caraballo *et al*., 2018), *HOX* and *COX-2* (Colón-Caraballo *et al*., 2015) genes, which seems to be the contradictory results considering endometriosis promoting roles of *ESR1* and *COX-2* and repressing roles of *PGR* and *HOX* (precisely *HOXA10*) genes. Nevertheless, reduced migration and proliferation followed by EZH2 pharmacological inhibition stresses its importance in endometriosis development and substantiates the need for further research.

CFP1 maintains the proper methylation, thus expression of downstream elements in PGR signalling. Its loss or downregulation can abolish the progesterone induced decidualization, causing embryo implantation disorders. This mechanism may contribute to infertility and endometriosis coincidence.

DNA modifications, predominantly cytosine methylation, introduced by enzymes, including DNMT and TET1, regulate expression of genes involved in pathological processes. Among discovered potential drivers of endometriosis development are: hypomethylation of coding region in CYP19A1 gene encoding aromatase (Izawa *et al*., 2011); homeobox A10 (HOXA10) promoter hypermethylation (Elias *et al*., 2023); estrogen receptor 2 (ESR2) (Xue *et al*. 2007a), steroidogenic factor-1 (SF-1) (Xue *et al*., 2007b) and cyclooxygenase-2 (COX-2) (Wang *et al*., 2012) promoter hypomethylation.

Non-coding RNAs, act on multiple stages including mRNA (microRNA and siRNA), protein-protein interactions (lnRNA) and RNA-protein interaction (lnRNA). Their influence on endometriosis development has been studied intensively in recent years. The levels of specific non-coding RNAs' sets in peripheral blood disclose a promising role in non-invasive endometriosis diagnosis. Moreover, the ability of these molecules to control specific genes expression and protein-protein interactions, makes them proper candidates for endometriosis therapies. The landscape of epigenetic changes responsible for endometriosis development presented in this paper can be targeted in such treatments. Combined with not invasive, therefore earlier, diagnosis non-coding RNAs can make a breakthrough in the field of endometriosis healthcare.

Whereas advancements have been made towards identifying epigenetic signatures and potential therapeutic targets, further investigations are warranted to elucidate the mechanistic underpinnings of epigenetic dysregulation in endometriosis. In the future larger cohort studies, interdisciplinary approaches, integrated multi-omics analyses and translational research efforts will be essential for improving outcomes for patients affected by endometriosis.

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List of abbreviations

5-caC – 5-carboxylcytosine
5-fC – 5-formylcytosine
5-hmC – 5-hydroxymethylcytosine
5-mC – 5C-methylocytosine
6-mA – N6-methyladenine
C – cytosine
CDHI – cadherine 1
COX-2 – cyclooxygenase-2
DNMT – DNA methylotransferase
DNMT1 – DNA methyl transferase 1
EMT – epithelial-to-mesenchymal transition
ERβ – estrogen receptor 2
ESR1 – estrogen receptor 1
EZH2 – homologue of zeste 2
G – guanine
GWAS – Genome Wide Association Study

H3 – histone 3
H3K27 – histone 3 on lysine 27
H3K27me3 – `3-methylated lysine 27 of histone 3
H3K4 – histone 3 on lysine 4
H3K9 – acetylation of histone 3 on lysine 9
H4 – histone 4
H4K16 – acetylation of histone 4 on lysine 16
HAT – histone acetylotransferase
HDAC – histone deacetylases
HDAC1 – histone deacetylase 1
HDAC2 – histone deacetylase 2
HMT – histone methyltransferase
HOXA10 – homeobox 10A
lncRNA – long non-coding RNA
LSD1 – histone demethylase 1
miRNA – microRNA
ncRNA – non-coding RNA
NGS – new generation sequencing
P4 – progesterone
PGR – progesterone receptor
RRM2–ribonucletide reductase subunit M2
SF-1 – steroidogenic factor-1
siRNA – small interfering RNA
VEGF – endothelial growth factor

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