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Review
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Review

DNA methylation alterations—potential cause of endometriosis pathogenesis or a reflection of tissue heterogeneity?†

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Abstract

Alterations in the DNA methylation pattern of endometriotic lesions and endometrium of endometriosis patients have been proposed as one potential factor accompanying the endometriosis development. Although many differentially methylated genes have been associated with the pathogenesis of this disease, the overlap between the results of different studies has remained small. Among other potential confounders, the impact of tissue heterogeneity on the outcome of DNA methylation studies should be considered, as tissues are mixtures of different cell types with their own specific DNA methylation signatures. This review focuses on the results of DNA methylation studies in endometriosis from the cellular heterogeneity perspective. We consider both the studies using highly heterogeneous whole-lesion biopsies and endometrial tissue, as well as pure cell fractions isolated from lesions and endometrium to understand the potential impact of the cellular composition to the results of endometriosis DNA methylation studies. Also, future perspectives on how to diminish the impact of tissue heterogeneity in similar studies are provided.

Summary Sentence

Cellular heterogeneity of endometriotic lesions and endometrial biopsies has significant impact on the results of DNA methylation studies in endometriosis.

Key words: endometriosis, epigenetics, DNA methylation, tissue heterogeneity, deconvolution.

Introduction

DNA methylation is a common epigenetic process, occurring mainly in CpG dinucleotide-rich areas, referred as CpG islands, where the cytosine nucleotide is converted by DNA methyltransferases into 5′-methylcytosine (5mC). Large majority of annotated gene promoters are associated with CpG islands and methylation of these islands may result in downregulation or silencing of the gene expression [1]. Thus, the DNA methylation has crucial role in regulation of many fundamental cellular processes, including genome regulation, development, and maintenances of tissue-specific gene expression pattern. Alterations disturbing DNA methylation/gene expression may initiate pathologic processes and contribute to the development of many diseases [2]. In recent years, alterations in the DNA methylation process have also been considered as one possible mechanism behind endometriosis development.

In addition, a functionally different type of DNA modification, namely hydroxymethylation, has recently been described [3,4]. Ten-eleven translocation (TET) proteins are responsible for creating 5-hydroxymethylcytosine (5hmC) [4] that is most abundant within the central nervous system, but has also been detected in other tissues including endometrium [5–8], and can contribute to the regulation of gene expression in endometriosis.

However, the number of studies evaluating the DNA methylation profiles in endometriosis has remained relatively moderate and only first attempts have been done to elucidate the possible role of hydroxymethylation in the disease development. To date, around 30 studies using both candidate gene-based and epigenome-wide association studies (EWAS) approaches have been conducted to uncover the DNA methylation changes in endometriotic lesions, in endometrium, and also in in vitro cultured endometrial stromal cells from lesions and endometrium (Tables 1–3). Despite the fact that these studies have brought out a large number of differentially methylated genes (e.g. Nuclear receptor subfamily 5 group A member 1 [*NR5A1*], Homeobox A10 [*HOXA10*], Progesterone receptor [*PGR*], *GATA binding protein 2 [GATA2*]) that could be associated with disease initiation, progression, and pathogenesis, the overlap between the results of different studies has remained small and alterations in DNA methylation patterns reported in one study are seldom confirmed by others. As the understanding about the nature of differential DNA methylation in different tissues and cell types is constantly evolving and there is clear evidence about the normal variability in DNA-methylation signature in different tissues and cells [9–11], it can be assumed that one potential reason for varying results of endometriosis studies may be hidden in the tissue/cell type heterogeneity (Figure 1). In endometriosis studies, different strategies have been applied and tissues with different level of cellular heterogeneity have been used. Some studies have compared whole-lesion biopsies with endometrial tissue; however, the cellular composition of endometriotic lesions is highly heterogeneous, as endometrial stromal and epithelial cells are mixed with cells from surrounding tissue (peritoneal tissue, ovarian components, etc.) and tissue-infiltrated blood cells in variable proportions. Therefore, the amount of disease-specific cells in lesions may have a crucial impact on the outcome of DNA methylation analysis and may lead to inconsistent or wrongly interpreted results. Some studies have analyzed moderately heterogeneous endometrial tissue, with no cellular contribution from other tissues, from patients and controls, and some have used pure endometrial stromal cell populations isolated from lesions and endometrium, as the study material with the lowest cellular heterogeneity. In this review, the results of DNA methylation and hydroxymethylation studies in endometriosis are discussed in the perspective of cellular heterogeneity, considering studies using high-, moderate-, and low heterogeneity samples.

High tissue/cellular heterogeneity—DNA methylation studies in endometriotic lesion whole-tissue biopsies

Highly heterogeneous endometriotic lesions, which are removed during laparoscopic surgery, have been an attractive study object for both candidate gene-based and genome-wide DNA methylation studies [12–26]. Up to date, DNA methylation profiles of more than 10 candidate genes and transposable elements involved in different pathways, such as hormonal signaling (*PGR*, Estrogen receptor 1 [*ESR1*], Estrogen receptor 2 [*ESR2*], prostaglandin-endoperoxide synthase 2, also known as COX-2 [*COX-2*], Catechol-O-methyltransferase [*COMT*]), ovarian cancer progression (Long interspersed nuclear element 1 [*LINE-1*]), carcinogenesis (Paired box 2 [*PAX2*]), tumor repressor and apoptosis-related genes (Cadherin 1 [*CDH1*], Ras association domain family [*RASSF*]), tissue remodeling (Matrix metallopeptidase 2 [*MMP2*], Matrix metallopeptidase 3 [*MMP3*], Matrix metallopeptidase 7 [*MMP7*], TIMP metallopeptidase inhibitor 3 [*TIMP3*], and TIMP metallopeptidase inhibitor 4 [*TIMP4*]), and genes needed for endometrial growth, differentiation, and implantation (*HOXA10*) have been investigated and associated with disease pathogenesis in whole endometriotic lesion biopsies (Table 1).

However, there are only two EWAS investigating DNA methylation in whole lesion tissues [24,25]. Borghese et al. [24] evaluated the DNA methylation status of more than 25,000 promoters using MeDIP-chip technology to ascertain the methylation profile of different type of endometriotic lesions (superficial endometriosis, ovarian cysts, and deeply infiltrating endometriosis). The study compared DNA methylation of pooled DNA samples from 15 eutopic endometria with pooled DNA samples from different lesion-types and detected 229, 161, and 108 differentially methylated regions in superficial endometriosis, ovarian cysts, and deeply infiltrating endometriosis, respectively. Some of the genes (*FLJ38379*, Defensin beta 125 [*DEFB125*], Golgin B1 [*GOLGB1*], *PERP*, TP53 apoptosis effector, nucleolar protein with MIF4G domain 1 [*NOM1*], centlein [*CNTLN*], *RASSF4*, *C10orf25*, *ZNF22*, HRas proto-oncogene, GTPase [*HRAS*], leucine rich repeat containing 56 [*LRRC56*], coagulation factor VII [*F7*], *DKFZp451A211*, ADP-ribosylhydrolase like 1 [*ADPRHL1*], tryptase delta 1 [*TPSD1*], pyrin domain containing 1 [*PYDC1*], testis expressed 14, intercellular bridge forming factor [*TEX14*], RAD51 paralog C [*RAD51C*], ring finger protein 126 [*RNF126*], follistatin like 3 [*FSTL3*], and ferritin heavy chain 1 pseudogene 19 [*FTHL19*]) were differentially methylated in all lesion subtypes. However, it should be pointed out that this work did not confirm the differential methylation of previously reported candidate genes. Authors suggested that this was because of the use of highly specific microarray with limited capacity to detect low CpG-containing regions. However, the most intriguing finding of this study was that in endometriotic lesions hypomethylated regions were distributed randomly across the chromosomes, whereas hypermethylated regions tended to locate at the ends of the

Table 1. Studies comparing DNA methylation between endometrium and endometriotic lesions.

Candidate gene studies			
Gene	Methylation status in lesion compared to endometrium	Region	Reference
COX-2	Hypomethylation	The NF-IL6 site within the promoter	[12]
COMT	Hypermethylation	Promoter region	[20]
CDH1	Hypermethylation	Promoter region	[18]
ESR1	ND	NA	[21]
ESR2	ND	NA	[21]
HOXA10	Hypomethylation	Two different promoter regions	[19]
HOXA10	Hypomethylation	Promoter region	[20]
LINE-1	Hypomethylation	NA	[13]
MMP2	Hypomethylation	Promoter region	[26]
MMP3, *MMP7*	Hypermethylation	Promoter region	[26]
PAX2	ND	Two different promoter regions	[16]
PGR	Hypermethylation	Promoter region B	[17]
PGR	ND	Promoter region A	[17]
PGR	Hypermethylation	Promoter regions A and B	[21]
RASSF1A	Hypermethylation	Promoter region	[15]
RASSF2	ND	NA	[14]
TIMP3	Hypermethylation	Promoter region	[26]
TIMP4	Hypermethylation	Promoter region	[26]
Epigenome wide association studies			
Cases/Controls (n)	**Results**	**Microarray**	**References**
Pooled samples of 5 OMA; 5 SUP; 5 DIE and15 E	229 DMRs in SUP, 161 DMRs in OMA, 108 DMRs in DIE	MeDIP array	[24]
8 OMA; 8 SUP; 8 E and 8 HE	3858 DMRs	Illumina Infinium HumanMethylation450 BeadChip	[25]

DIE, deep infiltrating endometriotic lesion; DMR, differentially methylated; E, endometriosis endometrium; HE, healthy endometrium; NA, not applicable; ND, no difference; OMA, endometrioma; SUP, superficial endometriotic lesion.

Table 2. Studies comparing DNA methylation in endometria of women with and without endometriosis.

Candidate gene studies			
Gene	Methylation status in endometria of patients compared to healthy endometria	Region	References
CDH1	Hypermethylation	Promoter region	[18]
COX-2	Hypomethylation	NF-IL6 site within the promoter	[12,38]
HOXA10	Hypomethylation	Promoter, intronic regions	[33]
HOXA10	Hypermethylation	Promoter	[19]
HOXA10	Hypermethylation	Promoter region	[36]
HOXA10	Hypermethylation	Upstream of exon 1, intron	[34,35]
HOXA11	Hypermethylation	Exon 1	[39]
PAX2	ND	Promoter	[16]
RASSF1A	Hypermethylation	Promoter region	[15]
RUNX3	Hypermethylation	Promoter region	[37]
Epigenome wide association studies			
Cases/controls (n)	**Results**	**Microarray**	**References**
7 E; 6 HE	120 DM genes	Illumina Infinium HumanMethylation27 BeadChip	[40]
31 E; 24 HE	28 DMRs	Illumina Infinium HumanMethylation450 BeadChip	[41]
17 E; 16 HE	MSE 137 CpG sites PE 58 CpG sites ESE 39 CpG sites	Illumina Infinium HumanMethylation27 BeadChip	[42]
8 E; 8 HE	ND	Illumina Infinium HumanMethylation450 BeadChip	[25]

DM, differentially methylated; DMR, differentially methylated region; E, endometriosis endometrium; ESE, early-secretory endometrium; HE, healthy endometrium; MSE, mid-secretory endometrium; ND, no difference; PE, proliferative endometrium.

Table 3. Methylation studies comparing endometriotic and endometrial stromal cells.

Candidate gene studies			
Gene	Methylation status of endometriotic stromal cells compared to endometrial stromal cells	Region	References
CYP-19	Hypomethylation	Exon 2	[48]
DUSP2	ND	Promoter region	[49]
ESR2	Hypomethylation	Promoter and exon 1 region	[81]
NR5A1	Hypermethylation	Intron 1	[46]
NR5A1	Hypermethylation	Exon 2 and intron 3 region	[45]
NR5A1	Hypomethylation	Promoter and exon 1 region	[81]
Epigenome wide association studies			
Cases/controls (n)	**Results**	**Microarray**	**References**
5 EcSCs; 5 ESC	43 DM genes	Illumina Infinium HumanMethylation450 BeadChip	[6]
6 EcSCs; 6 ESC	9021 DM genes	Illumina Infinium HumanMethylation450 BeadChip	[50]
3 EcSCs + 3 ENSC; 3 ESC	770 DM genes	Illumina Infinium HumanMethylation27 BeadChip	[52]

DM, differentially methylated; EcSCs, ectopic endometrial stromal cells; ENSC, endometrial stromal cells from women with endometriosis; ESC, endometrial stromal cells from healthy women; ND, no difference.

chromosomes. The authors proposed that noticed asymmetric methylation pattern enhances chromosome stability and presumes endometriotic cells from malignant transformation [24]. In the most recent study, Rahmioglu et al. [25] analyzed the DNA methylation profiles of 14 endometriotic lesions (both endometriomas and peritoneal lesions) and 16 endometrial samples from endometriosis patients and found 27,493 significantly differentially methylated sites corresponding to 8133 genes. Among these genes was a significant enrichment for *WNT* signaling, angiogenesis, cadherin signaling, and gonadotropin-releasing-hormone-receptor pathways that have been previously associated with endometriosis pathogenesis. The authors brought out that endometrium, endometriomas, and peritoneal lesions have their distinct DNA methylation signatures and suggested that whole-tissue profiling will detect robust DNA methylation between the individuals, but for low-variability DNA methylation sites the cellular heterogeneity and technical variability hinder the detection of biologically meaningful alterations. Furthermore, significant impact of menstrual cycle phases on endometrial and peritoneal lesions DNA methylation signature was noticed, but in endometriomas, this effect was less pronounced [25].

The studies concerning hydroxymethylation level in endometriosis whole tissues have given contradictory results, most probably because of different methodologies used to detect the amount of 5hmc. Using an ELISA-based colorimetric quantification, it was found that DNA of whole endometriotic tissues contains very high amounts of 5hmC compared to nonendometriosis control eutopic endometrium, a phenomenon corroborated by the inverse expression of TET genes in these tissues [5]. In the following immunofluorescence study, Yotova et al. showed that on the whole tissue level there was a disease-dependent loss of 5hmC in the endometriotic tissue epithelial cells but not in the stromal cell compartment [6].

The high heterogeneity of endometriotic lesions' cellular composition and lack of knowledge about the normal DNA methylation profiles of surrounding tissues and cells makes the discovery of disease specific changes extremely difficult and even if thousands of differentially methylated genes have been reported, it is almost impossible to distinguish whether these findings reflect the complex mixed cellular composition of lesions or these alterations are truly endometriosis-related changes.

Moderate tissue/cellular heterogeneity—DNA methylation studies of endometrium and potential impact of menstrual cycle on endometrial methylome

The theory of endometrial origin of endometriosis, which postulates that endometrial cells are refluxed via retrograde menstruation and implant into the abdominal cavity and form ectopic lesions, is widely accepted [27]. However, for successful establishment of lesions, endometrial cells of endometriosis patients should have altered characteristics triggering the endometrial cell adhesion and growth in the ectopic locations. This assumption is supported by multiple lines of evidence showing that endometria of endometriosis patients have aberrant gene expression profiles compared to healthy women [28–30]. As DNA methylation is a potential cause of gene expression alterations, it has encouraged researchers to seek for the methylation changes in patient's endometria. Although the endometrium is a mixture of different cell types, where endometrial apical and glandular epithelial and stromal cells are mixed with tissue-infiltrated blood cells, the cellular heterogeneity between endometrial samples from patients and controls is definitely less pronounced than between endometrium and lesion biopsies, facilitating the identification of true disease-related DNA methylation changes. Still, the search for disease-related endometrium-specific DNA methylation alterations is a challenging task because of extensive molecular, morphological, and physiological changes occurring during the menstrual cycle. Furthermore, the impact of menstrual cycle phases on the DNA methylation profile in healthy women's endometrial tissue has recently been demonstrated [31, 32].

The candidate gene-based and DNA microarray studies comparing endometria from women with and without endometriosis have brought out number of disease-related genes (Table 2) [12,15,16,18,19,33–42]. The candidate gene-based studies have reported dysregulation of genes involved in many important biological functions, such as hormonal regulation (*COX-2*), development of female genital tract (Paired box 2 [*PAX2*]), endometrial growth and receptivity (*HOXA10*, *HOXA11*), and tumor suppression (Runt related transcription factor 3 [*RUNX3*], *RASSF1A*, *CDH1*) [12,33,34,36,38,39,43,44].

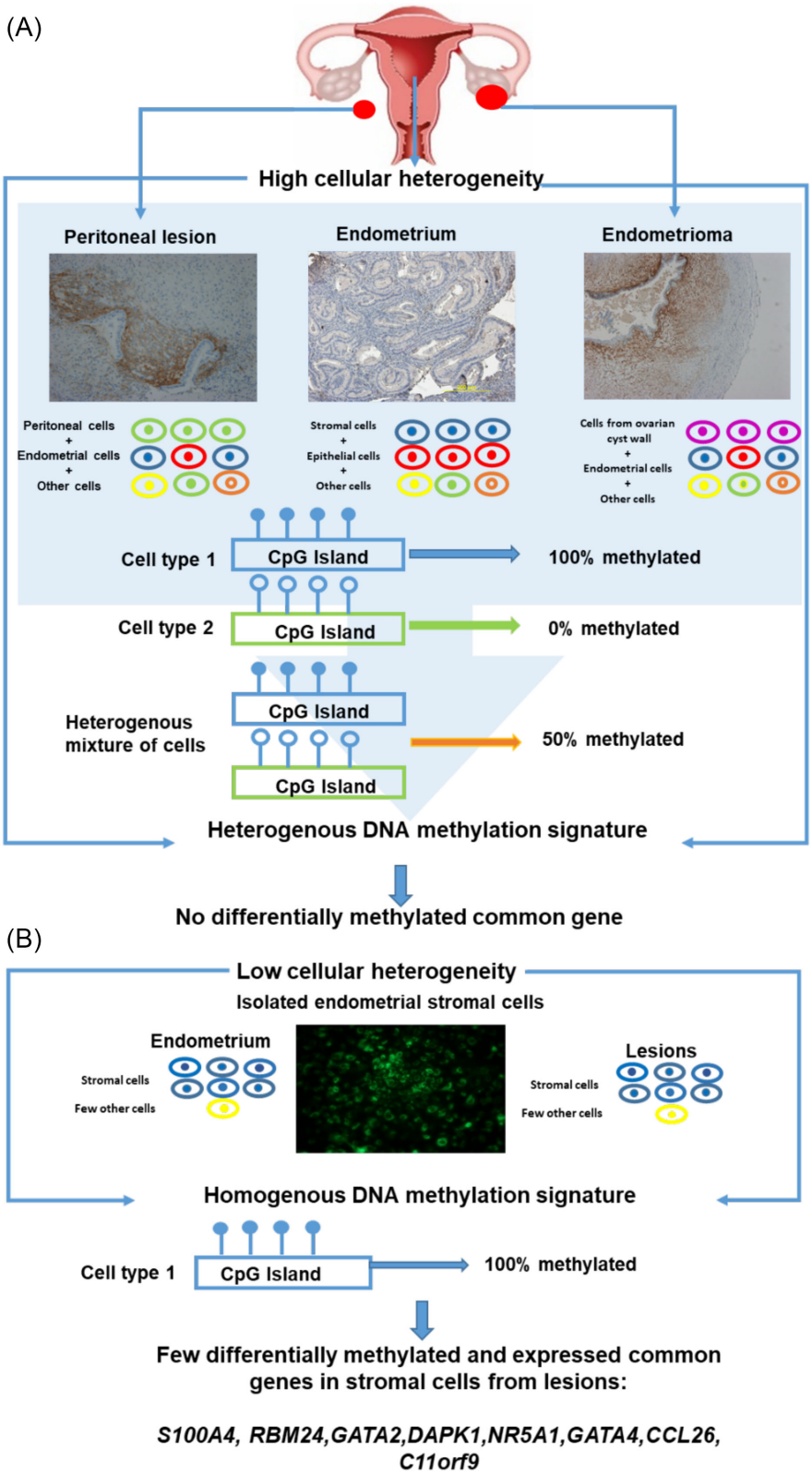

Figure 1. The impact of tissue heterogeneity on endometriosis DNA methylation studies. (A) Peritoneal lesions and endometriomas contain only a small fraction of endometrium-specific cells (brown color in endometrioma and peritoneal lesion histological section indicates $CD10^{+}$ endometrial stromal cells that surround the endometrial epithelial glandular structure) surrounded by other cell types. This may lead to a heterogeneous DNA methylation signature as the same CpG sites may have a cell-type specific methylation status. (B) Stromal cells isolated from endometrioma and endometrium are with low cellular heterogeneity (immunofluorescence staining of cultured stromal cells using antibody against CD10), and the obtained DNA methylation signature is characteristic to a homogeneous cell population.

In addition to candidate gene studies, four EWAS comparing endometria from healthy women and endometriosis patients have been published [25,40–42]. The study by Naqvi et al. [40] investigated 27,578 CpGs in 7 women with endometriosis and 6 controls and found 120 statistically significant differentially methylated genes (59 hypermethylated and 61 hypomethylated). They also confirmed correlations between methylation status and gene expression level among a set of 10 selected genes and proposed that these genes may contribute to the abnormal regulation of endometrial cell proliferation in women with endometriosis. However, our recent study suggested that methylation profiles between endometria of patients (n = 31) and controls (n = 24) are highly similar [41]. We found only 28 differentially methylated regions, from which 16 were associated with known genes—Peptidase inhibitor 3 (*PI3*), solute carrier family 43 member 3 (*SLC43A3I*), mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase, isozyme B (*MGAT5B*), mucin 4, cell surface associated (*MUC4*), human immunodeficiency virus type I enhancer binding protein 3 (*HIVEP3*), fibrinogen gamma chain (*FGG*), cardiotrophin like cytokine factor 1 (*CLCF1*), calcium activated nucleotidase 1 (*CANT1*), leukocyte receptor tyrosine kinase (*LTK*), aryl-hydrocarbon receptor repressor (*AHRR*), aldo-keto reductase family 1 member B (*AKR1B1*), acylaminoacyl-peptide hydrolase (*APEH*), cystatin 11 (*CST11*), ELOVL fatty acid elongase 4 (*ELOVL4*), hemoglobin subunit epsilon 1 (*HBE1*), and neuronal growth regulator 1 (*NEGR1*). Furthermore, the overall magnitude of methylation differences was rather small, and as the biological significance of small-scale changes in DNA methylation levels is currently unknown, it is unclear whether and how these changes are associated with disease pathogenesis. In addition, methylation status of some of these genes, such as *PI3, SLC43A3I*, *MGAT5B*, *MUC4*, *HIVEP3*, *FGG*, *CANT1*, and *LTK*, was influenced by menstrual cycle phase, indicating that it is crucially important to take into account the normal epigenetic changes across the menstrual cycle when looking for disease specific methylation differences in endometrial tissue [41]. The menstrual cycle-dependent endometrial DNA methylation was also confirmed by Houshdaran et al. [42], who found that the DNA methylome differences between the endometriosis patients (n = 17) and controls (n = 16) were most contrasting in the mid-secretory phase, at the time of the progesterone peak (137 CpG sites, corresponding to 125 loci), followed by proliferative (58 CpG sites, corresponding to 58 loci) and early-secretory phase (39 CpG sites, corresponding to 36 loci). Moreover, if all patient samples were compared to all control samples regardless the menstrual cycle phase, only three differentially methylated loci (ribosome production factor 2 homolog [*RPF2*], period circadian regulator 1 [*PER1*], and family with sequence similarity 181 member A [*FAM181A*]) remained. Locus in gene *RPF2* was more methylated in controls, while loci in *PER1* and *FAM181A* were more methylated in endometriosis patients [42]. Menstrual cycle phase as an important covariate in DNA methylation and RNA expression analysis was also reported by Rahmioglu et al. [25], who found no significant changes between the endometrial DNA methylation profiles of women with and without endometriosis but reported significant variability of DNA methylation between the different menstrual cycle phases.

While we compared all available data from the findings of genome-wide studies demonstrating any changes in endometrial DNA methylation in endometriosis [40–42], we found no common genes, indicating that DNA methylation changes in endometrium are probably not the main trigger leading to endometriosis development. On the other hand, it is possible that the confounding factors, like menstrual cycle, study design, differences in data analysis, and interpretation, mask small but relevant changes. Furthermore, power calculations revealed that most of the so far performed studies have been unpowered to detect reliable methylation differences between the groups [25]. For example, to detect 2% ($\Delta\beta = 0.02$) DNA methylation difference between cases and controls, at least 500 patient's samples are needed [25].

Low tissue/cellular heterogeneity—DNA methylation studies of endometrial primary stromal cells

Investigation of primary cell cultures enables to minimize the confounding effects of the accompanying cells in the tissues and to compare the same type of cells irrespective of the original location. The use of primary cells from lesions and endometrium in DNA methylation studies has its own pros and cons as discussed below, but still offers a good solution to study the molecular mechanisms of endometriosis with minimal impact of cellular heterogeneity.

To date, there have been nine studies (six candidate-gene and three EWAS) determining the DNA methylation profiles of primary stromal cells from endometrium and lesions [45–51,6,52] (Table 3). Closer look at the candidate-gene studies reveals that all studies have been focused on the genes participating in the hormonal regulation of the endometrial cells, such as *NR5A1*, *ESR2*, Cytochrome P450 family 19 (*CYP19*), and dual specificity phosphatase 2 (*DUSP2*). The most commonly studied gene *NR5A1* [45–47] encodes transcription factor steroidogenic factor 1 (SF-1) that has a role in activating *STAR* and aromatase production and therefore could potentially contribute to the higher level of estrogens in endometriotic stromal cells [53]. Interestingly, methylation status of distinct regions of *NR5A1* influences its expression in different ways. Hypermethylation of the proximal promoter of *NR5A1* in endometrial stromal cells is accompanied by the lack of *NR5A1* expression, and on the opposite, the hypomethylation of the same region in endometriotic cells leads to the high expression of *NR5A1* mRNA, confirming the general understanding that DNA methylation of promoter region is inversely correlated with transcription [47]. However, the following studies revealed that hypermethylation of CpG islands in introns and exon 2 leads to the high expression of *NR5A1* mRNA in endometriotic cells [46,54] indicating that methylation of CpG islands outside the promoter region may also play an important role in regulating *NR5A1* expression.

Beside candidate-gene studies, three EWAS methylation studies have been conducted concentrating on stromal cells from ovarian cysts and eutopic endometria from endometriosis patients and healthy women. As a result, a large number of potentially disease-related differentially methylated genes were detected (9021, 770, and 43, respectively) [50,6,52]. When we compared the lists of genes that were reported in all three studies, only a small subset of overlapping genes (S100 calcium binding protein A4 [*S100A4*], RNA binding motif protein 24 [*RBM24*], GATA binding protein 2 [*GATA2*], death associated protein kinase 1 [*DAPK1*], *NR5A1*, *C11orf9*, C-C motif chemokine ligand 26 [*CCL26*], and GATA binding protein 4 [*GATA4*]) was found. Some of the genes from this list are particularly interesting. For example, the GATA family members have previously been associated with induction of "ovarian-like" differentiation of ectopic endometrial cells [55]. It has been suggested that similarly to the ovarian cells, the expression of *GATA4* and *GATA6* transcription factors is induced via the positive feedback loop by the follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) genes also in ectopic endometrial cells. Thus, the

parallel increase in GATA4/6 transcription factors and FSHR and LHR levels leads to enhanced production of the steroidogenic cascade that supports estrogen-dependent disease progression [55]. Furthermore, the hypermethylation and downregulation of *GATA2* strongly regulates the genes essential for decidualization, and hypomethylation and activation of *GATA6* promotes an endometriotic phenotype through progesterone resistance and altered estrogen response [50]. Also, all microarraybased studies have reported hypermethylation of fibroblast-specific protein-1 (*S100A4*). The expression of this gene is shown to be induced by the TGF-β pathway, and silencing of *S100A4* expression can inhibit the process of TGF-β-induced epithelial-mesenchymal transition (EMT) [56]. As during the EMT process epithelial cells lose their specific features and acquire more invasive mesenchymal characteristics, the EMT is believed to be a crucial event leading to the development of endometriosis [57].

It is worth mentioning that the differential methylation and expression of *NR5A1* seen in candidate-gene studies was proven in EWAS studies. Moreover, it was confirmed that not only the promoter-region hypomethylation regulates the expression of this gene [50,52] but also that the intronic region hypermethylation in endometriotic stromal cells allows for higher level of *NR5A1* expression [50].

The first study assessing the role of 5hmC in endometrial and endometriotic stromal cells [6] demonstrated that detected altered methylation of brain derived neurotrophic factor (*BDNF*) gene in the EWAS was partly caused by hydroxymethylation [6]. Still, to understand the exact role of 5hmC in endometriosis development and endometrial biology, future studies elucidating the changes of 5hmC in different cell types obtained from the endometrium or endometriotic lesions and along the menstrual cycle are urgently needed.

The studies on isolated stromal cells have found the greatest number of overlapping differentially methylated and expressed genes, demonstrating the benefit of investigating homogenous cell populations. However, it should be pointed out that the use of primary cells from lesions and endometrium in DNA methylation studies has its own strengths and weaknesses. On the one side, primary cells are phenotypically and epigenetically similar to the same cell type in the tissue of origin, but on the other side, the natural environment of the cells is destroyed in cellular separation with cells adapting quickly to in vitro conditions. The in vitro environment with supporting hormonal milieu is especially important in case of hormone-dependent cells such as endometrial cells. Furthermore, primary cell culturing and repeated passaging changes the cell subpopulation dynamics and leads to alterations in their whole transcriptome [58,59] and in DNA methylation [60]. Thus, to keep the molecular signatures as similar as possible to the original tissue, the studies of the primary cells should be limited only to the early passages [58,59]. Furthermore, although it is generally accepted that primary cell culture is a homogenous mixture of identical cells, most of the primary cultures also include a small fraction of other cell types from the same tissue, ranging usually around 1%–5% [61–64]. The issue of cellular contamination becomes particularly important for establishing slowly proliferating epithelial cell culture as even a small contamination with highly proliferative stromal cells may largely change the cellular composition of the culture. Although large efforts have been made to culture primary endometrial epithelial cells from endometrium, these cells can only be passaged once [65,66] with cell senescence becoming evident already within 2 weeks in culture [67]. Therefore, as culturing of endometrial epithelial cells has still remained a challenge, no specific studies on DNA methylation alterations in endometrial epithelial cells from endometriotic lesions exist.

Moreover, in case of isolation and culturing of primary endometrial stromal cells from endometriomas, it is impossible to identify the possible ovarian stromal contamination due to the lack of specific markers [68], creating additional bias in endometrioma studies. Last but not least, DNA methylation studies on isolated stromal cells of lesions have thus far been concentrated only on cells isolated from endometriomas. Whether there are also DNA methylation alterations in cells isolated from peritoneal lesions is currently unknown but definitely worth to study. Therefore, in order to eliminate the bias coming from cell culturing and potential contamination of the primary cell culture, we suggest using uncultured cells from lesions and endometrium to reveal the true molecular differences occurring inside the lesions, as discussed in the next section.

Possible approaches to diminish cellular heterogeneity in endometriosis studies

To diminish cellular heterogeneity in endometriosis studies and analyze DNA methylation of endometrium-specific cells with minimal impact of cells from surrounding tissues, several solutions could be proposed. First, specific cell populations can be isolated from histological tissue sections, such as formalin-fixed and paraffin-embedded (FFPE) tissues, RNAlater stored, or snap-frozen tissues, by laser capture microdissection (LCM). Although this methodology has been suggested to be time-consuming, labor-intensive, and providing only limited amount of DNA for methylation analysis, it still represents the most effective technology to isolate a morphologically homogeneous population of specific cells. However, the attractiveness of this methodology has remained small and to date, there are no DNA methylation studies in endometriosis that had used the potential advantage of LCM. It is very likely that small interest of using LCM for isolation of specific cell populations is related to the poor quality of DNA obtained, especially when the DNA is isolated from archived FFPE tissues. For example, LCM DNA from snap-frozen sections allows to amplify DNA regions of >300 bp, while from FFPE tissues only 150 to 200 bp fragments are available [69]. However, cancer studies have clearly proven the usefulness of LCM in DNA methylation analysis [70].

Second, the uncultured single cells or cell populations from lesion biopsies and endometrium can be isolated using fluorescently labeled antibodies against cell surface markers in combination with fluorescence activated cell sorting (FACS). FACS methodology has previously been used to isolate specific cell types from endometrium of healthy women [71] and from endometrium of women suffering from endometriosis [72,73] for gene expression studies. We have shown the usefulness of this methodology for isolation of CD10-positive stromal cells from endometrioma biopsies for transcriptome study [72] and the amount of cells obtained by FACS should also be suitable for DNA methylation analysis. Currently, the main limitation of this methodology is the absence of specific antibodies to discriminate endometrial epithelial cells from lesion biopsy. While the anti-CD10 antibody discriminates CD10-negative ovarian stromal cells from CD10-positive endometrial stromal cells [74], the previously used endometrial epithelial cell-specific anti-CD9 antibody [71] does not distinguish them from similar CD9-positive granulosa and epithelial cells from endometrioma samples [75]. Furthermore, it should be kept in mind that beside endometrial epithelial and stromal cells, lesions also contain other cell types and DNA methylation changes in these cells may have significant impact on disease pathogenesis and deserve further investigation.

Thirdly, computational approaches for tackling cellular heterogeneity can also be used. As already stated, each tissue or cell type has a specific methylation signature and fluctuations in biopsy cellular composition can dramatically confound analyses, either by creating false-positive associations (e.g. the detected differences actually simply tag the change in cellular composition) or masking actual associations. To overcome this obvious limitation in EWAS, several computational approaches have been developed, which allow us to adjust for cellular heterogeneity, an approach known as cell-type deconvolution (reviewed by [76]). In broad terms, these deconvolution methods can be either reference-based or reference free. The former approach uses reference methylation profiles for cell types present in the tissue and works under the assumption that the methylation profile of a whole-tissue biopsy is a sum of reference profiles of each cell type present in this biopsy. As a result, the algorithm calculates the fractions for each cell type that match the whole-tissue biopsy profile best. These fractions can then be used as covariates in differential methylation analysis to adjust for differences in whole-tissue biopsy cellular composition. A major drawback for using this approach in the context of endometriosis studies is the lack of suitable reference profiles for cell types present in the endometrial tissue or endometriotic lesions.

As an alternative, reference-free deconvolution methods such as EWASher [77], RefFreeEWAS [78], and ReFACTor [79] or surrogate variable analysis (SVA) that allow to adjust for fluctuations in cellular composition can be used. Selection of the best method depends on study design and research question, but SVA was shown to be the most stable and robust method across different scenarios [80]. However, currently no study has compared the performance of these different methods in the context of endometriosis. Therefore, since it is clear that tissue heterogeneity is a very important confounder in endometriosis research, there is a critical need to describe accurately the cellular (sub)populations present in endometrium and endometriotic lesions to generate high-quality reference methylomes for these cell types, and to compare the performance of different deconvolution algorithms for endometrium and endometriotic lesions. These bits would provide the necessary basis to tackle the cellular heterogeneity on a computational level and provide a viable alternative to analyzing separate cell populations, which is labor-intensive and costly.

Conclusions

The first steps to unravel the role of altered DNA methylation and hydroxymethylation in endometriosis development have been done but there is a long way to go before we can ascertain whether the knowledge gained from these studies could be benefitted in improving the endometriosis diagnostics or therapy. To overcome the issues related to tissue biopsy heterogeneity, the methylation differences should be demonstrated in pure populations of cells and a direct link between the methylation and gene expression alterations in the same cells should be established.

Declaration of Conflicting Interests

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

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