Endometriosis Is Characterized by a Distinct Pattern of Histone 3 and Histone 4 Lysine Modifications

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Abstract

Background: The histone modification patterns in endometriosis have not been fully characterized. This gap in knowledge results in a poor understanding of the epigenetic mechanisms (and potential therapeutic targets) at play. We aimed to (1) assess global acetylation status of histone 3 (H3) and histone 4 (H4), (2) measure levels of H3 and H4 lysine (K) acetylation and methylation, and (3) to identify histone acetylation patterns in promoter regions of candidate genes in tissues from patients and controls. **Methods:** Global and K-specific acetylation/methylation levels of histones were measured in 24 lesions, 15 endometrium from patients, and 26 endometrium from controls. Chromatin immunoprecipitation (ChIP)–polymerase chain reaction was used to determine the histone acetylation status of the promoter regions of candidate genes in tissues. **Results:** The lesions were globally hypoacetylated at H3 (but not H4) compared to eutopic endometrium from controls. Lesions had significantly lower levels of H3K9ac and H4K16ac compared to eutopic endometrium from patients and controls. Tissues from patients were hypermethylated at H3K4, H3K9, and H3K27 compared to endometrium from controls. The ChIP analysis showed hypoacetylation of H3/H4 within promoter regions of candidate genes known to be downregulated in endometriosis (e.g., *HOXA10*, *ESR1*, *CDH1*, and *p21^{WAF1/Cip1}*) in lesions versus control endometrium. The steroidogenic factor 1 (SF1) promoter region was enriched for acetylated H3 and H4 in lesions versus control tissues, correlating with its reported high expression in lesions. **Conclusions:** This study describes the histone code of lesions and endometrium from patients with endometriosis and provides support for a possible role of histone modification in modulation of gene expression in endometriosis.

Keywords

endometriosis, epigenetics, histone code, histone modifications, histone acetylation, histone methylation, ChIP

Introduction

Endometriosis, the growth of endometrial-like cells outside the uterus, is thought to occur due to differential regulation of gene expression in ectopically growing tissues.¹ Epigenetic mechanisms have been shown to be responsible, at least in part, for the differential global gene expression profiling seen in endometriotic lesions and in endometrium of patients with endometriosis.^{2–4} Epigenetics, the stable inheritance of a phenotype without changes in the DNA sequence, refer to regulatory mechanisms, including methylation of CpGs in promoter regions, micro RNA-mediated transcription regulation, and covalent histone modifications also known as “histone marks.”⁵ Covalent modifications of histones, which include acetylation, methylation, and phosphorylation, are mediated by the balanced interplay of enzymes such as histone deacetylases (HDACs), histone acetylases (HATs), and histone demethyltransferases (HDMTs).^{6,7} Recently, strong evidence for a role of histone acetylation in endometriosis has

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been published, including the observation of a higher expression of HDACs in endometriotic tissues and the ablation of the endometriotic phenotype in vitro and in vivo by HDAC inhibition (HDACi), although the specific mechanisms involved are still under investigation.⁸⁻¹¹ These studies are of high translational relevance since HDACi has been proposed as a possible therapeutic strategy for endometriosis based on its effects on cell proliferation and apoptosis.⁴

There is increasing evidence to suggest the existence of a “histone code,” the specific histone modification pattern that characterizes a particular differentiation or pathological state.^{12,13} This complex language consists of specific modifications at histone tails (e.g., histone marks) that have regulatory effects in a small number of target genes.¹⁴⁻¹⁶ It is now widely accepted that this is an important biological phenomenon resulting in gene deregulation and disease; therefore, efforts are in place to help decipher this code, as it relates to complex conditions, such as cancer,^{17,18} autoimmune disorders,¹⁹⁻²¹ and chronic pelvic pain.²² Histone acetylation is generally associated with activation of gene expression and histone methylation with gene silencing. By causing changes in chromatin structure, and thereby providing or blocking access of reader/effector modulators and of transcription factors to their binding sequences in gene promoters, different combinations of these modifications help orchestrate gene expression that can lead to disease.²³

Based on recent reports from our laboratory and others showing that elevated levels of HDACs may play a role in endometriosis, we hypothesized that endometriotic lesions would be characterized by a hypoacetylated phenotype at H3 and H4, specifically near or within the promoter regions of candidate genes, and by a specific profile or signature of specific histone marks that could explain the reported high-/low-transcriptional activity of candidate genes. The objective of this study was, thus, to determine global acetylation status of histones in endometriotic lesions and to identify the histone code that characterizes ectopically growing endometrium. In addition, we aimed to determine the acetylation status of promoter sequences of candidate genes (*HOXA10*, *ESR1*, *CDH1*, *p21*, and steroidogenic factor 1 [*SF-1*]) in eutopic endometrium and endometriotic tissues. Quantification of acetylated histone levels and specific lysine (K) acetylation and methylation levels is a first, important step in the efforts to dissect the mechanisms of epigenetic regulation of gene expression and will contribute to the development of epigenetic-based therapies for endometriosis. This study described the histone code of lesions and endometrium from patients with endometriosis which may play a pathological role via regulation of gene expression leading to activation of the disease mechanisms. In addition, we show that the promoters of candidate genes for endometriosis are characterized by a distinct pattern of histone acetylation in lesions compared to eutopic endometrium from a reference group that correlates with their reported gene expression pattern in lesions. Thus, these data provide additional support for a role of histone modification in endometriosis and a possible therapeutic target for this disease.

Materials and Methods

Tissue Collection

Fresh endometriotic lesions ($n = 24$) and endometrial biopsies ($n = 38$) were obtained from women undergoing surgery for benign gynecologic conditions including endometriosis at 3 collaborating hospitals. A team of gynecologists validated the diagnosis of endometriosis or the absence of disease by visual inspection of the pelvis during surgery using revised American Society for Reproductive Medicine (rAFS) criteria and completing a standard surgery report. The average age of patients (30.9, range 16-48 years) and controls (36.1, range 21-47 years) was significantly different ($P = .046$). In the chromatin immunoprecipitation–polymerase chain reaction (ChIP-PCR) studies, the average age of patients with and without endometriosis was 35.2 years (range: 26-47 years) and 33.7 years (range: 23-43 years), respectively. The reference group included women with a diagnosis of uterine fibroids, primary dysmenorrhea, adhesions, or multiparity who were surgically confirmed of not having endometriosis. Gynecologic and demographic profiles of patients in the study are summarized in Tables 1 and 2. All frozen tissues were evaluated pathologically to confirm diagnosis and determine menstrual cycle phase of endometrial tissues according to Noyes.²⁴ An hematoxylin and eosin–stained guide slide was used by the pathologists to mark areas containing glands and stroma. After macrodissection to exclude nonendometriotic tissue (i.e., adjacent peritoneum), the tissues were stored at -80°C until analysis. All protocols involving tissue collection were approved by the Institutional Review Board Committee of Ponce School of Medicine and Health Sciences (PSMHS). All patients donating tissue for these studies signed a consent form and completed a demographic, gynecologic, and clinical questionnaire.

Total Protein Purification and Histone Extraction

Tissue fragments were weighed and cut into small pieces (1-2 mm³) with a scalpel before homogenization with a Dounce homogenizer. The homogenate was resuspended in lysis buffer containing 2% sodium dodecyl sulfate in phosphate-buffered saline (PBS) and supplemented with proteinase and phosphatase inhibitors. The total protein concentration was quantified using Bio-Rad protein assay (Valencia, California). Bovine Serum Albumin (BSA) was used to generate a standard curve. To identify histone modifications, nuclear proteins were extracted following the manufacturer's detailed protocol (EpiQuick Nuclear Extraction kit, Epigentek, Brooklyn, New York).

Detection of Global Acetylation Status of H3 and H4

Assessment of global H3 and H4 acetylation (H3ac, H4ac) was conducted using the EpiQuick Global Histone H3/H4 Acetylation Assay Kit (Epigentek) following the manufacturer's protocol. The H3-specific immunoassay detects acetylated K9 and K14 residues, while the H4-specific assay detects acetylation status of H4K5, K8, K12, and K16, thus providing a global score of K acetylation. In brief, protein samples (200 ng/ μL) were added to the wells on

Table 1. Demographic Characteristics of Study Patients.

Pt ID	Diagnosis	Age	ASRM Stage	Tissue	Reg Cycle	Phase
268	Endometriosis	35	III-IV	Ovarian endometriosis	Yes	Secretory
822	Endometriosis	30	I-II	Superficial peritoneal lesion	Yes	Secretory
854	Endometriosis	28	III-IV	Ovarian endometriosis	Yes	Secretory
866	Endometriosis	32	I-II	Superficial peritoneal lesion	Yes	Proliferative
901	Endometriosis	44	I-II	Superficial peritoneal lesion	Yes	Proliferative
1008	Endometriosis	42	III-IV	Ovarian lesion		
1018	Endometriosis	16	I-II	Cul de sac lesion	Yes	Proliferative
1025	Endometriosis	37	III-IV	Ovarian lesion	Yes	Proliferative
1056	Endometriosis	48	I-II	Ovarian lesion	Yes	Secretory
1066	Endometriosis	22	I-II	Superficial peritoneal lesion	No	Proliferative
1068	Endometriosis	17	III-IV	Deep peritoneal lesion	No	Secretory
1080	Endometriosis	20	I-II	Superficial peritoneal lesion	Yes	Proliferative
1084	Endometriosis	42	I-II	Superficial peritoneal lesion	Yes	Secretory
1097	Endometriosis	18	I-II	Superficial peritoneal lesion	Yes	Secretory
1125	Endometriosis	47	III-IV	Fallopian tube endometriosis	Yes	Secretory
805	Endometriosis	25	I-II	Endometrium	Yes	Proliferative
815	Endometriosis	28	I-II	Endometrium	Yes	Proliferative
833	Endometriosis	41	I-II	Endometrium	Yes	Proliferative
834	Endometriosis	36	I-II	Endometrium	Yes	Proliferative
839	Endometriosis	30	I-II	Endometrium	Yes	Proliferative
889	Endometriosis	28	I-II	Endometrium	Yes	Secretory
904	Endometriosis	38	I-II	Endometrium	Yes	Secretory
907	Endometriosis	36	I-II	Endometrium	Yes	Proliferative
920	Endometriosis	32	I-II	Endometrium	Yes	Secretory
924	Endometriosis	38	I-II	Endometrium	Yes	Secretory
947	Endometriosis	22	I-II	Endometrium	Yes	Proliferative
964	Endometriosis	28	III-IV	Endometrium	Yes	Secretory
968	Endometriosis	17	I-II	Endometrium	Yes	Unknown
974	Endometriosis	41	I-II	Endometrium	Yes	Secretory
980	Endometriosis	32	III-IV	Endometrium	Yes	Proliferative
987	Endometriosis	34	I-II	Endometrium	No	Secretory
994	Endometriosis	34	I-II	Endometrium	Yes	Secretory
804	Iry dysmenorrhea	32		Endometrium	Yes	Secretory
821	Spontaneous miscarriages	29		Endometrium	Yes	Secretory
829	Spontaneous miscarriage	42		Endometrium	Yes	Proliferative
905	Endometriosis	38		Endometrium	Yes	Secretory
954	Fibroids	37		Endometrium	Yes	Proliferative
963	CPP	44		Endometrium	Yes	Secretory
966	Fibroids	45		Endometrium	Yes	Proliferative
975	Fibroids	43		Endometrium	Yes	Secretory
983	Fibroids	32		Endometrium	Yes	Proliferative
986	Metrorrhagia	35		Endometrium	No	Unknown
991	Fibroids	47		Endometrium	Yes	Secretory
998	Fibroids	47		Endometrium	Yes	Proliferative
1019	Ovarian tumor	43		Endometrium	No	Unknown
1058	CPP	27		Endometrium	Yes	Proliferative
1095	Iry dysmenorrhea	21		Endometrium	Yes	Secretory

Abbreviation: ASRM, American Society for Reproductive Medicine.

the EpiQuick 96-well plate and incubated according to the manufacturer's instructions. Colorimetric analysis was performed using a microplate reader. A standard curve was generated by plotting the optical density values of a dilution series made from a 100% acetylated protein standard (supplied with the kit), the slope of which was used to calculate the total amount of acetylated protein in each sample. The amount of acetylated protein in nanogram per milligram was obtained by dividing the acetylated protein amount by

the total protein input amount divided by the slope. At least 2 technical replicates were conducted for validation purposes.

Detection of Specific K Acetylation or Pan-Methylation at H3 and H4

Next, we used EpiQuick immunoassay kits (Epigentek) specific for 4 histone K acetylations (H3K9ac, H4K5ac, H4K8ac,

Table 2. Demographic Characteristics of Patients in ChIP Studies.

ID	Sample	Diagnosis		Age	ASRM Stage	Regular Cycle	Phase
1	Control	Pelvic adhesions	1058	27	N/A	Y	P
2	Control	Ovarian tumor	1019	43	N/A	N	P
3	Control	Metrorrhagia	804	32	N/A	Y	S
4	Control	Primary Dysmenorrhea	986	35	N/A	N	U
5	Control	Myomatous uterus	975	43	N/A	Y	S
6	Control	Dysmenorrhea	1052	33	N/A	Y	S
7	Control	Myomatous uterus	991	47	N/A	Y	S
8	Control	Myomatous uterus	966	45	N/A	Y	P
9	Control	Miscarriages	821	29	N/A	Y	S
10	Control	Myomatous uterus	1101	39	N/A	Y	S
11	Lesion	Ovarian endometriosis	854	28	III/IV	Y	S
12	Lesion	Ovarian endometriosis	741	44	III-IV	Y	S
13	Lesion	Ovarian endometriosis	268	35	III/IV	Y	S
14	Lesion	Ovarian endometriosis	208	32	III/IV	N	P
15	Lesion	Peritoneal endometriosis	1084	42	III/IV	Y	S
16	Lesion	Cul de Sac endometriosis	506	46	I-II	Y	U
17	Lesion	Ovarian endometriosis	658	45	III/IV	Y	P
18	Lesion	Ovarian endometriosis	1011	35	I-II	Y	S
19	Lesion	Fallopian tube endometriosis	1128	28	III-IV	Y	S
20	Lesion	Cul de Sac endometriosis	288	30	I-II	Y	P
21	Lesion	Fallopian tube endometriosis	1125	47	III/IV	Y	S

Abbreviations: ASRM, American Society for Reproductive Medicine; ChIP, chromatin immunoprecipitation; N, no; N/A, not available; P, proliferative; S, secretory; U, unknown; Y, yes.

and H4K16ac) and 3 histone K pan-methylations (H3K4me, H3K9me, and H3K27me). These histone marks were selected based on the fact that they are the most widely studied modifications and on the availability of commercial immunoassays. Panmethylation refers to the fact that this assay does not distinguish between the 3 levels of methylation known, monomethyl (me1), dimethyl (me2), or trimethyl (me3). Two technical replicates were conducted for validation purposes.

Statistical Analyses

Nonparametric analysis of variance (ANOVA) with Dunn posttest was conducted to determine statistical significance of differences among the study groups using GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, California). Statistical significance was set at $P < .05$. Receiver–operating characteristic (ROC) analyses were conducted to assess the predictive value of selected histone marks in endometrium from patients and controls (eg, specificity and sensitivity, likelihood ratio).

Tissue ChIP

The ChIP assay was performed using the EpiQuik Tissue Chromatin Immunoprecipitation kit (Epigentek). Endometriotic ($n = 11$) and reference eutopic endometrial ($n = 10$) tissues were weighed and cut into small pieces (1–2 mm³) with scissors. Frozen tissues were transferred to a conical vial and cross-linked with 1% formaldehyde in the Roswell Park Memorial Institute medium. The reaction was stopped with 1.25 mol/L glycine. The mix was centrifuged, the supernatant was removed, and the pellet was washed with 10 mL ice-cold

PBS. Another centrifugation was done, and the tissue pieces were transferred to a Dounce homogenizer and 1 mL of homogenizing buffer was added per every 200 mg of tissue. Tissue pieces were disaggregated by 10 to 20 strokes. The disaggregated tissue pellet was incubated in lysis buffer containing protease inhibitors, followed by DNA sonication. After centrifugation (14 000 rpm for 10 minutes), an aliquot of the supernatant was incubated for 2 hours with anti-H3 (1:50) and anti-H4 (1:25; Cell Signaling Technology, Boston, Massachusetts) antibodies previously cross-linked with the 96-well strips. An aliquot of each supernatant was used as the input control. Positive and negative controls were processed using anti-RNA polymerase II and normal mouse immunoglobulin G, respectively. The immune complexes and input controls were incubated with proteinase K at 65°C, transferred to the column, washed with 70% and 90% ethanol, and finally the purified DNA was eluted.

Polymerase Chain Reaction Amplification

Primer pairs of promoter regions of candidate genes described in Table 3 were designed using the Basic Local Alignment Search Tool/National Center for Biotechnology information program of the GenBank and Primer3 (v.0.4.0) software. In addition, the alignments of the primers were made using Align sequence W2 EBI software. The PCR mixture contained 1× PCR buffer with 15 mmol/L MgCl₂ (16.6 mmol/L ammonium sulfate), 25 mmol/L MgCl₂, 1× Q-solution, deoxynucleotides (each at 300 μmol/L), primers (0.5 μmol/L each per reaction), 1 unit of Taq Polymerase, and DNA (25 ng) in a final volume of 50 μL. Amplification was carried out in a PTC-200 Pelter

Table 3. Oligonucleotides Used for ChIP-PCR.

Primer Set	Sequence 5' to 3'	Size, bp	Anneal Temperature, °C
SF-1	F-AGTCTCTGCTCCGTCTTGTA R-AATCTCGCCTGCGTTGTAGT	928	62
HOXA10	F-CTTACCCACCGTGTGTGTTG R-TGTCTGCGTGTCTGCCTATC	821	55.2
CDH1	F-AGTCCCACAACAGCATAGGG R-TGGGGTCTCACTCTTTCACC	921	56.2
ESR1	F-GCCCCATTCTACCATTCTCA R-CCAGGCCCGAATCTAACTTT	678	55.6
p21	F-GGAGGCAAAAGTCCTGTGTT R-GGCTCCACAAGGAAGTACT	1025	55.6

Abbreviations: bp, base pair; ChIP-PCR, chromatin immunoprecipitation–polymerase chain reaction; SF-1, steroidogenic factor 1; HOXA10, Homeobox A 10; CDH1, E-cadherin; ESR-1, estrogen receptor.

Thermal Cycler (MJ Research, Waltham, Massachusetts) for 29 cycles of 95°C for 30 seconds, 30 seconds at the annealing temperature listed in Table 1, and 40 seconds at 72°C followed by a final 5-minute extension at 72°C. Controls without DNA were performed for each set of PCRs. Each PCR (10 µL) was loaded onto 3% agarose gels, stained with ethidium bromide, and directly visualized under ultraviolet illumination using the ChemiDoc XRS+ (BioRad, Hercules, California).

Results

Endometriotic Lesions Are Characterized by Global H3 Hypoacetylation

We measured concentration and quantified global K acetylation at H3 and H4 in endometriotic tissues and eutopic endometrium from patients with and without endometriosis. As shown in Figure 1, the lesions were globally hypoacetylated at H3 when compared to most endometrium from patients without endometriosis, which could be categorized in 2 groups, hypo- and hyperacetylated at H3. This difference was not statistically significant; however, after removing the samples showing a hypoacetylated phenotype at H3 (box), the difference reached statistical significance (ANOVA $P = .0015$; Figure 1A). No differences were observed among groups in their global acetylation levels at H4 (Figure 1B).

Endometriotic Lesions Are Characterized by Hypoacetylation at H3K9 and H4K16

The average total acetylation levels of H3K9 (H3K9ac) were significantly lower in endometriotic lesions when compared to eutopic endometrium from both patients with endometriosis ($P < .01$) and from controls ($P < .001$; ANOVA, $P = .0001$; Figure 2). Average total acetylation levels of H4K16 (H4K16ac) were significantly lower in lesions when compared to eutopic endometrium from both patients with endometriosis and controls ($P = .0001$; Figure 3). No significant differences were observed either for the H4K5ac or the H4K8ac marks

after 1 trial, and therefore these histone marks were not studied further (data not shown).

Endometriotic Lesions and Endometrium From Patients With Endometriosis Are Characterized by H3K4 Hypermethylation

The average total methylation levels of H3K4 (H3K4me) were significantly different among groups (ANOVA $P = .0001$) and were highest in lesions, intermediate in endometrium from patients, and lowest in endometrium from patients without endometriosis (Figure 4). The ROC analysis was conducted to determine the predictive value of H3K4me levels in the endometrium of patients with endometriosis. A threshold value of >993.5 mg/protein of H3K4me was able to differentiate endometriosis with 95.65% sensitivity and 93.33% specificity (area under the curve = 0.9478; $P < .0001$) and a likelihood ratio of 14.35.

Endometriotic Lesions and Endometrium From Patients With Endometriosis Are Characterized by H3K9 Hypermethylation

The average total methylation levels of H3K9 (H3K9me) were lower in control endometrium when compared to tissues from patients with endometriosis (both lesions and eutopic endometrium; ANOVA $P = .0015$; Figure 5). The ROC analysis was conducted to determine the predictive value of H3K9me levels in the endometrium of patients with endometriosis. A threshold value of >720.6 mg/protein of H3K9me was able to differentiate endometriosis with 66.67% sensitivity and 66.67% specificity ($P = .001257$) and a likelihood ratio of 2.0.

Endometriotic Lesions and Endometrium From Patients With Endometriosis Are Characterized by H3K27 Hypermethylation

The average total methylation levels of H3K27 (H3K27me) were significantly different among groups (ANOVA $P = .0001$) and

Figure 1. Global acetylation of histones 3 and 4 in endometriosis and endometrium from patients and controls. Global acetylation levels were determined using the EpiQuick Global Histone H3/H4 Acetylation Assay kit (Epigentek, Brooklyn, New York). A standard curve was generated by plotting the optical density (OD) values of a dilution series made from a 100% acetylated protein standard, the slope of which was used to calculate the total amount of acetylated protein in each sample. The amount of acetylated protein in nanogram per milligram was obtained by dividing the acetylated protein amount and the total protein input amount, divided by the slope. Endometriotic lesions were hypoacetylated at H3 (analysis of variance $P < .0015$; lesions vs endometrium from controls $P < .01$; A). H4 acetylation levels were not different among groups (B). Experiments were conducted at least twice, and data are graphed as box-and-whisker plots (line = median; whiskers = minimum to maximum). Endometriotic tissues ($n = 14$), endometrium from women with endometriosis ($n = 9$), and endometrium from controls ($n = 15$).

were significantly higher in both lesions and endometrium from patients with endometriosis when compared to endometrium from patients without endometriosis (Figure 6). The ROC analysis was conducted to determine the predictive value of H3K27me levels in the endometrium of patients with endometriosis. A threshold value of >956.1 mg/protein of H3K27me was able to differentiate endometriosis with 95.24% sensitivity and 93.33% specificity ($P = .001257$) and a likelihood ratio of 14.29. Figure 7 shows that there were no significant differences in the histone marks studied based on menstrual cycle phase.

Global H3/H4 Acetylation Status of Candidate Gene Promoters in Endometriotic and Endometrial Tissues

The ChIP-PCR analyses were conducted to examine the global levels of H3 and H4 acetylation in the promoter regions of selected genes, known to be differentially expressed in tissues from endometriosis patients. These analyses identified distinct patterns of histone acetylation at H3 and H4, which correlated with the reported levels of expression of these genes in endometriotic lesions (Figure 8). Data are summarized in Table 4. Of note, lesions with hyperacetylated H4 near/within the promoter of *HOXA10* were ovarian lesions (5 of 6) and 1 cul-de-sac lesion. The only 3 lesions with deacetylated H4 near/within the promoter of *SF-1* were peritoneal (2) and cul-de-sac (1). Lesions with hyperacetylated H3 near/within the promoter were mostly ovarian (5 of 6).

Discussion

Epigenetic mechanisms have recently been shown to be involved in the pathophysiology of endometriosis; however, data are lacking on the role of histone modifications in this disease.⁴ The present study was conducted to contribute to this emerging field by conducting a comprehensive and specific assessment of the acetylation and methylation status of selected lysine residues of H3 and H4 in endometriotic and endometrial tissues obtained from patients with and without endometriosis. Also, we aimed to determine whether the global histone acetylation status of selected candidate gene promoter regions would differ in endometriotic and endometrial tissues from patients within these 2 groups. We showed that endometriotic lesions had lower global acetylation levels of H3 (but not of H4) compared to endometrium from most patients without endometriosis. We also observed significant differences in the levels of specific histone modifications in tissues obtained from patients with endometriosis, which has been referred to as histone code. These findings have important implications for the diagnosis and treatment of this enigmatic disease.²⁵ Specifically, it is highly relevant to decipher the endometriosis-specific histone code, since inhibitors of HDACs and other epigenetic modulators,²⁶ are being considered as therapeutic target for endometriosis.⁴

Covalent modification of histone tails is regarded as an important mechanism that contributes to the coordinated regulation of genes involved in differentiation, cell cycle, and carcinogenesis, among many other cellular processes.²⁷⁻³⁰ Acetylation

Figure 2. The H3K9 acetylation levels in endometriosis and endometrium from patients and controls. Acetylation levels were determined using the EpiQuick H3K9 Acetylation Assay kit (Epigentek, Brooklyn, New York). The amount of acetylated protein in ng/mg of protein was obtained by dividing the acetylated protein amount and the total protein input amount, divided by the slope. Average total acetylation levels of H3K9 (H3K9ac) were significantly lower in endometriotic lesions when compared to both eutopic endometrium from patients ($P < .01$) and from controls ($P < .001$); ANOVA $P = .0001$). Endometriotic tissues ($n = 14$), endometrium from women with endometriosis ($n = 9$), and endometrium from controls ($n = 15$). Experiments were conducted at least twice, and data are graphed as box-and-whiskers plots (line = median; whiskers = minimum to maximum). ANOVA indicates analysis of variance; H3, histone 3; K, lysine; ac, acetylation.

of lysine residues is the first well-studied histone modification and has been correlated with transcriptional activation via mechanisms related to either direct effects on the chromatin structure and/or recruitment of effector/reader modules or transcription factors.^{31,32} There is increasing evidence showing that distinct global histone acetylation levels characterize diseased states.^{26,33,34} Also, it has been shown that the genes involved in ovarian steroid hormone function, inflammation, and cell cycle control, including 2 of the genes studied here, *ESR1* and *p21^{WAF1/CIP1}*, are transcriptionally modulated by acetylation of histones.³⁵⁻³⁹ However, very few studies have been conducted on endometriosis in this regard.⁴⁰

As a first step in dissecting the role of this epigenetic mechanism in endometriosis, we quantified the global acetylation levels of H3 and H4 in ectopic and eutopic endometrial tissues from patients with and without endometriosis. We hypothesized that lesions will have globally deacetylated histones. This hypothesis was based on several observations. Guo and colleagues showed that HDACi was able to revert the phenotype of endometriotic cells in vitro, and of lesions in vivo, making these cells less invasive, less proliferative, and lesions smaller.^{9,41} We extended

Figure 3. H4K16 acetylation levels in endometriosis and endometrium from patients and controls. Acetylation levels were determined using the EpiQuick H4K16 Acetylation Assay kit (Epigentek, Brooklyn, New York). The amount of acetylated protein in nanogram per milligram was obtained by dividing the acetylated protein amount and the total protein input amount divided by the slope. Average total levels of H4K16ac were significantly lower in endometriotic lesions when compared to eutopic endometrium from both patients and controls ($P = .0001$). Endometriotic tissues ($n = 14$), endometrium from women with endometriosis ($n = 9$), and endometrium from controls ($n = 15$). Experiments were conducted at least twice, and data are graphed as box-and-whisker plots (line = median; whiskers = minimum to maximum). H4 indicates histone 4; K, lysine; ac, acetylation.

those observations by showing that HDAC1 and HDAC2, two of the most widely studied HDACs, were aberrantly expressed in endometriotic tissues.¹¹ These data suggested that a dysregulated deacetylase activity in ectopically growing endometrium would result in hypoacetylation of histones and in specific long-term transcriptional effects leading to the endometriotic phenotype.^{12,42,43} We observed differences in global acetylation levels of H3 (but not in H4) in endometriotic lesions when compared to endometrium from patients with other gynecologic conditions. We next asked whether specific K residues of H3 and H4 could be hypoacetylated and also investigated the methylation status of these 2 histone tails. Our aim was to identify a signature consisting of covalent modifications of histones (ie, histone marks) which when present in specific combinations could modulate the expression of selected genes in a way that may result in the endometriotic phenotype.⁴⁴ We observed that endometriotic

Figure 4. H3K4 methylation levels in endometriosis and endometrium from patients and controls. Methylation levels were determined using the EpiQuick H3K4 Methylation Assay kit (Epigentek, Brooklyn, New York). Amount of methylated protein in nanogram per milligram was obtained by dividing the methylated protein amount and the total protein input amount divided by the slope. Average total methylation levels of H3K4 (H3K4me) were significantly different among groups (ANOVA $P = .0001$) and were highest in lesions, intermediate in endometrium from patients, and lowest in endometrium. Endometriotic tissues ($n = 14$), endometrium from women with endometriosis ($n = 15$), and endometrium from controls ($n = 15$). Experiments were conducted at least twice, and data are graphed as box-and-whisker plots (line = median; whiskers = minimum to maximum). ANOVA indicates analysis of variance; H3, histone 3; K, lysine; me, methylation.

Figure 5. H3K9 methylation levels in endometriosis and endometrium from patients and controls. Methylation levels were determined using the EpiQuick H3K9 Methylation Assay kit (Epigentek, Brooklyn, New York). The amount of methylated protein in nanogram per milligram was obtained by dividing the methylated protein amount and the total protein input amount divided by the slope. Average total methylation levels of H3K9 (H3K9me) were lower in control endometrium versus tissues from patients with endometriosis (ANOVA $P = .0015$). Endometriotic tissues ($n = 14$), endometrium from women with endometriosis ($n = 15$), and endometrium from controls ($n = 15$). Experiments were conducted at least twice, and data are graphed as box-and-whisker plots (line = median; whiskers = minimum to maximum). ANOVA indicates analysis of variance; H3, histone 3; K, lysine; me, methylation.

lesions are characterized by hypoacetylation at H3K9 and H4K16 and hypermethylation at H3K4, H3K9, and H3K27.

The H3K9ac, one of the best studied histone marks, has been associated with transcriptionally active chromatin.⁴⁵⁻⁴⁸ Several genes related to endometriosis, including *p16*, *MLH1*, and *HOX*, have been shown to be regulated by H3K9ac.^{49,50} Interestingly, levels of H3K9ac decreased from benign hyperplasia to intraepithelial neoplasia to adenocarcinoma,⁵¹ and higher H3K9ac levels have been associated with better prognosis.⁵²⁻⁵⁴ Levels of the H3K9ac mark have been shown to increase after treatment with HDACi.⁵⁵ The known roles for H3K9ac in stem cell differentiation and cancer make it a relevant histone mark in the context of endometriosis and other endometrial disorders.^{56,57}

The H4K16ac is regarded as a positive modulator of gene expression. Hypoacetylated H4K16 appears early and accumulates during tumor development in an in vivo model of carcinogenesis, suggesting that loss of H4K16 acetylation is necessary for malignant transformation.⁵⁸ The H4K16ac was found to be at low or undetectable levels in the majority of breast tumor cases on a TMA.⁵⁹ The important roles of this histone mark in carcinogenesis, silencing of tumor suppressor genes, cellular life

span, and activation of antiapoptotic genes also make this mark one of the high relevance to the endometriosis phenotype.⁶⁰

We also studied selected histone methylation marks and observed that tissues from women with endometriosis, both lesions and endometrium, were characterized by increased levels of methylation at H3K4, H3K27, and H3K9, which were significantly different from those obtained from women with other gynecologic conditions. Methylation at H3K4 is a well-known epigenetic mark associated with transcriptional activation and is correlated with poor prognosis and recurrence of cancer.^{61,62} Methylation at H3K27 is one of the main repressive histone modifications and has been linked to polycomb-group-protein-mediated suppression of *HOX* genes.⁶³ This mark has also been correlated with severity and histological differentiation of cancer.^{17,63} When occurring together, the repressive H3K27me mark and the activating H3K4me mark constitute a “bivalent domain”; in this mode developmental genes such as *HOXA11* in stem cells are silenced but primed for quick activation.^{64,65} The H3K9me together with H3K27me negatively correlates with gene expression of target genes.⁶⁶ There are other combinations of histone marks shown to have distinct, reproducible effects across systems.²³ The H3K9ac, H4K16ac,

Figure 6. H3K27 methylation levels in endometriosis and endometrium from patients and controls. Methylation levels were determined using the EpiQuick H3K27 Methylation Assay kit (Epigentek, Brooklyn, New York). The amount of methylated protein in nanogram per milligram was obtained by dividing the methylated protein amount and the total protein input amount divided by the slope. Average total methylation levels of H3K27 (H3K27me) were significantly different among groups (ANOVA $P = .0001$) and were significantly higher in both lesions and endometrium from patients with endometriosis versus endometrium from controls. Endometriotic tissues ($n = 14$), endometrium from women with endometriosis ($n = 15$), and endometrium from controls ($n = 15$). Experiments were conducted at least twice, and data are graphed as box-and-whisker plots (line = median; whiskers = minimum to maximum). ANOVA indicates analysis of variance; H3, histone 3; K, lysine; me, methylation.

and H3K4me are markers for gene activation⁶⁷; H3K4me in combination with H4K16ac or H3K9/14/18/23ac leads to transcription activation; jointly, H3K27me and H3K4me play fundamental roles in regulation of developmental genes. As research efforts continue to understand this code, and the “meaning” of the different combinations of histone marks is uncovered, the histone code of endometriosis will be better understood.

Histone modifications are increasingly being recognized as key factors in gene regulation in a variety of diseases including gynecologic cancers, but there have been very few investigations on their possible role in normal endometrium and endometrial pathologies such as endometriosis.^{6,29,68,69} A recent study that compared global H3 and H4 acetylation status and H3K4 and H3K9 methylation in lesions versus endometrium from healthy women showed what can be perceived as different results from those presented here; however, data sets are difficult to compare due to lack of detail in technical and analytical methods as well as due to differences in the reference group.⁴⁰ A study by Munro et al investigated the global histone

acetylation levels in normal endometrium during the menstrual cycle.^{31,70} They reported that acetylation levels of some but not all histone acetylation marks were increased in the early proliferative phase and/or during the window of implantation, when active transcription of genes would be expected. Also, acetylation of selected Ks in H3 and H4 has been shown to be modulated in response to ovarian steroid hormones.⁷¹ However, we did not observe significant differences among the 5 histone marks studied according to menstrual cycle phase, perhaps because our study only looked at proliferative versus secretory endometrium and did not consider subphases of the cycle. Little is known about hormonal regulation of histone tail methylation and demethylation, and the enzymes involved have only recently been discovered.⁷² Notably, histone marks have been shown to be biochemically stable and to be transmitted transgenerationally, which could explain the lack of cycle dependency of the histone marks studied.⁷³⁻⁷⁵ Also, it can be speculated that loss of regulation in histone modification in response to environmental stimuli (such as ovarian hormones) is the underlying mechanism of epigenetic diseases.⁷⁶

Using ChIP, we also assessed the H3 and H4 global acetylation patterns associated with the promoter regions of candidate genes (*HOXA10*, *ESR1*, *CDH1*, *p21*, and *SF-1*) and observed differences in endometriosis lesions compared to control tissues (endometrium from patients without endometriosis). Histone deacetylation correlated with reported gene expression profiles of the genes studied. We also observed differences in acetylation pattern associated with the promoter region according to the localization of the lesions for several of the genes studied.

Homeobox A 10 (*HOXA10*), a transcription factor involved in endometrial development during menstrual cycle and implantation of the embryo,^{77,78} has been found to be hypermethylated in the endometrium of women with endometriosis when compared to endometrium of women free of disease. It has been suggested that *HOXA10* may have an important role in regulating endometrial receptivity, and defects in its expression may reduce fertility.⁷⁹ We observed that endometriotic lesions are globally hypoacetylated at H3 near the *HOXA10* promoter. This is in accord to the previously shown observation that H3K9 was hypoacetylated leading to a reduced transcription at *HOX* loci.⁵³

We observed that the *CDH1* promoter is hypoacetylated at both histones H3 and H4 in endometriotic lesions when compared to controls. Previous studies showed that trichostatin A treatment induces BMP-7 which, in turn, increases E-cadherin expression, suppressing epithelial-to-mesenchymal transition in renal epithelial cells.⁸⁰ Also, the promoter of *CDH1* was found to be hypermethylated in endometriosis, and treatment with an HDAC inhibitor reactivated its expression. Together, these data suggest that histone deacetylation works in concert with DNA methylation to suppress the expression of *CDH1* in endometriosis.

It has been previously shown that in endometriosis there are decreased levels of *ESR1*, and epigenetic mechanisms have been implicated in the disruption of the *ESR1-ESR2* ratio.⁸¹

Figure 7. Histone acetylation and methylation levels are not significantly different according to the menstrual cycle phase. Data were analyzed by t test to determine whether the levels of the histone marks studied varied according to menstrual cycle phase. Endometriotic tissues (n = 14), endometrium from women with endometriosis (n = 15), and endometrium from controls (n = 15). Experiments were conducted at least twice, and data are graphed as box-and-whisker plots (line = median; whiskers = minimum to maximum).

Interestingly, the *ESR1* promoter was associated with H3ac in most of the ovarian lesions studied, but not in the peritoneal lesions, which may suggest a differential role of this epigenetic mechanism in different lesion types.

The *p21^{WAF1/Cip1}* gene encodes a cell cycle kinase inhibitor associated with cell cycle control. It has been shown that HDACi induces *p21^{WAF1/Cip1}* expression and cell cycle arrest in endometrial stromal cells.⁹ Acetylation of H3 is associated with *p21^{WAF1/Cip1}* expression in cancer,⁴² and treatment with HDACi causes its reactivation in colon cancer cell lines. This gene has been implicated in endometriosis; however, little is known about its regulation. We observed that the *p21^{WAF1/Cip1}* promoter was hypacetylated at both the histones in endometriotic lesions when compared to control tissues.

Finally, *SF-1* is a transcription factor critical in the activation of multiples genes in estrogen biosynthesis, including aromatase. The *SF-1* is reported to be overexpressed in endometriotic stromal cells⁸² due to promoter hypomethylation, and, interestingly, we show here that there is an enrichment of acetylated H3 and H4 in this promoter that occurs specifically in endometriotic lesions. These data suggest that acetylation of histones near the

SF-1 promoter may contribute to its aberrant expression in lesions, contributing to overexpression of aromatase and local estradiol.

It is important to note that histone modification is a highly complex and evolving field of study, and the regulatory network that controls this epigenetic mechanism is still not fully understood.⁴ These results must be expanded into studies of other types of histone modifications, levels of K methylation (mono-, di-, tri-, each one with a different outcome), and to H1 and H2, which were not studied here. Other limitations of these studies include the fact that we analyzed whole tissues, which are composed of a mixed population of cells (stromal, epithelial, and inflammatory) that may contribute differently to the global acetylation and methylation status of the histone studied. Although methods are available for studying the stromal and epithelial components separately (e.g., microdissection), in actuality these methodologies are limited in the amount of tissue that is isolated which may affect the type of downstream analysis that can be conducted. On the other hand, it can be argued that it is of great interest to better understand what is going on in the lesions in vivo and to consider the outcomes of cell-cell interactions. Ultimately, it would be important to assess

Figure 8. Global H3/H4 acetylation status of candidate gene promoter regions in endometriotic and endometrial tissues. Chromatin immunoprecipitation–polymerase chain reaction (ChIP-PCR) was conducted using primers specific for promoter region of selected candidate genes on immunoprecipitated DNA protein samples obtained from lesions (n = 11) and endometrium from controls (n = 10). Antiacetylated H3 (H3ac) and antiacetylated H4 (H4ac) were used for immunoprecipitation. DNA indicates deoxyribo nucleic acid; IgG, immunoglobulin G; H3, histone 3; H4, histone 4; K, lysine; ac, acetylation; me, methylation. IgG negative control; RNA Pol II positive control.

Table 4. Summary of ChIP Analysis of Global H3 and H4 Acetylation.

Gene	ID	Expression in Endometriosis	Results of Global Acetylation of H3 and H4 in Lesions vs Control Endometrium
Homeobox A 10	<i>HOXA10</i>	Downregulated	All lesions were deacetylated at H3 when compared to controls; 5 of 11 lesions were deacetylated at H4
Estrogen receptor α	<i>ER1</i>	Downregulated	H3 and H4 were deacetylated in 6 and 7 of the total of 11 lesions, respectively; controls were all acetylated.
E-cadherin	<i>CDH1</i>	Downregulated	H3 and H4 were globally deacetylated in all endometriotic lesions compared to controls
p21	<i>p21^{WAF1/Cip1}</i>	Downregulated	H3 and H4 were globally deacetylated in the endometriotic lesions compared to controls
Steroidogenic factor 1	<i>SF-1</i>	Upregulated	H3 was acetylated in all endometriotic lesions; H4 was acetylated in 8 of the 11 lesions

Abbreviations: ChIP, chromatin immunoprecipitation; H3, histone 3; H4, histone 4.

the different combinations of histone marks and the synergism with other epigenetic and genetic mechanisms in order to better understand the etiopathology of endometriosis.⁸³ This knowledge can then be used to design ways of regulating epigenetic states leading to novel therapeutic options for endometriosis.

In sum, our results extend prior studies of histone acetylation status in endometriosis and endometrium, respectively, and suggest that histone modifications may play an important role in the development of this disease, as it has been shown for other complex conditions. These data contribute to a better

understanding of how patterns of gene expression are modulated in endometriosis via mechanisms that include enzymatic setting of specific patterns of histone modifications, involving not only global acetylation but also acetylation and methylation of particular K residues. Moreover, these data are highly relevant for the development of potential new drugs that target the epigenetic mechanisms at play in endometriosis.

Authors' Note

The first author JBM conducted most of the experiments reported, including macrodissection, protein extraction of tissues, histone mark analyses as well as data analysis and interpretation. She was primarily involved in writing the first draft of the manuscript. The second author MC-D helped with tissue processing, histone extraction, conducted the ChIP experiments reported, and helped with data analysis and manuscript writing. The authors MG and SG were in charge of the pathological evaluation of tissues used in this study, including validation of the endometriosis histological diagnosis (glands and stroma) and selection of areas for macrodissection. MC helped with tissue processing, histone extraction, and the ChIP experiments. ES provided key expertise in epigenetics of cancer, helped with data interpretation, and conducted critical review of the manuscript. JL, the director of the Ob-Gyn Department at PSMHS, provided support in the clinical aspects of the study, helped with identification of study participants, tissue accrual, and validation of diagnosis. IF is the principal investigator of this study and the director of the Endometriosis Research Program at PSM. She was primarily responsible for overseeing all aspects of this study, from patient recruitment to final data analysis. She was involved in editing and revision of the manuscript, preparation of figures, and overseeing all statistical analysis.

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