HDAC class IIa expression and regulation in human endometrial tissues and stromal cells during the menstrual cycle

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Research Article

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Abstract

Background: Histone Deacetylases (HDACs) are a class of enzymes that deacetylate the lysine residues on the protruding histone tails, causing tightening of the chromatin structure and making genes inaccessible to be transcribed. Class I and II HDACs have been involved in cell cycle regulation and progression. Class 1 HDACs have been widely studied in endometrial pathologies and menstrual cycle regulation. Studies have shown that class IIa HDACs (4, 5, 7, and 9) regulate the cell cycle, proliferation, and differentiation. But any potential roles for class IIa HDACs in endometrial biology have not been explored. This study aims to characterise the temporal expression of class IIa HDACs in the endometrium and their regulation by steroid hormones in human endometrial stromal cells (HESCs).

Methods: mRNA expression and protein expression of HDAC 4, 5, 7, and 9 were analyzed in endometrial tissue biopsies collected from premenopausal women throughout the menstrual cycle by real-time quantitative PCR and fluorescent western blot analysis, respectively. Effect of hormonal regulation on class IIa HDAC expression was studied in endometrial stromal cell line following treatments with estrogen (E2) and estrogen-progesterone combined (E2+P4) over 24hrs, 48hrs, and 72hrs period. The cells were also treated with HDAC inhibitor Trichostatin A (TSA) combined with the steroid hormones.

Results: In endometrial tissue, mRNA expression of class IIa HDACs showed cyclic changes. *HDACs 5* and *7* were significantly upregulated during the early secretory phase, while HDAC9 was upregulated during the mid-secretory phase, compared to other cycle stages. In addition, HDAC 5 and 9 proteins were significantly upregulated in the human endometrium during the secretory phase, compared to the proliferative stage. Protein expression levels of class IIa HDACs in HESCs were upregulated in response to the combined estrogen-progesterone treatment for 24 and 48 hrs, *in vitro*. While TSA mitigated these hormonal effects..

Conclusions: This study shows that class IIa HDACs are expressed in the human endometrium in a cyclic pattern and are influenced by steroid hormones. Suggesting their possible involvement in menstrual cycle regulation and endometrial pathologies.

Introduction

The human endometrium is a steroid hormone-sensitive tissue that lines the uterus and provides an optimal environment for embryo implantation [1]. In the first half of the cycle, the rise in estrogen levels stimulates proliferation and primes the endometrium for progesterone action and secretory changes, enriching it with steroid hormone receptors. This phenomenon of estrogen priming is crucial for proper response to progesterone and optimal differentiation in the second half of the cycle. Post-ovulation, the rise in progesterone together with estrogen initiates secretory modifications and stromal cell decidualization [2]. Genes associated with these changes are epigenetically regulated by several factors, particularly DNA methylation and histone acetylation [3-8].

Histone acetylation is one of the most studied histone modifications controlled by two opposing sets of enzymes - Histone acetyltransferases (HATs) and Histone deacetylases (HDACs). HATs and HDACs co-regulate gene expression and maintain endometrial function with other regulators [3, 9]. The HDACs are involved in post-translational modifications of protein and essentially act to remove acetyl groups from proteins. Removal of acetyl groups from the lysine residues of histone proteins changes the conformation of chromatin structure and making transcription factor binding sites inaccessible [10, 11]. There are 18 known HDACs found in humans, divided into four classes. Class I (HDAC 1, 2, 3, and 8); class II (HDAC 4, 5, 6, 7, 9 and 10); class III (SIRT 1, 2, 3, 4, 5, 6 and 7) and class IV (HDAC 11) [10, 12].

Class 1 HDACs are one of the most studied HDAC classes in endometrium and have been heavily implicated in many endometrial pathologies [13-16]. In addition, they are known to be associated with steroid hormone-dependent gene expression and are crucial in maintaining the health and function of the endometrium [13, 16]. However, Class IIa HDACs, have not been entirely explored yet in the endometrium.

Class IIa HDACs possess additional C-terminal nuclear export signals, which enable translocalization between the nucleus and cytoplasm. The presence of both nuclear localization signal (NLS) and a nuclear export signal (NES) helps them to compartmentalize themselves accordingly [17], giving them the unique ability to readily travel between nuclear and cytoplasmic compartments in response to post-translational modifications [18, 19]. Since HDACs' ability to silence genes epigenetically is dependent on their presence in the nucleus, their cellular localization can be used to predict the deacetylation ability of chromatins. Furthermore, nuclear export prevents class IIa HDACs from acting as transcriptional repressors, thus resulting in inducible gene expression [20]. In some cases, class IIa HDACs can also act as transcriptional activators by binding to inhibitory domains of genes. However, it is evident that these enzymes primarily control gene expression by recruiting other proteins (corepressors or coactivators) [19, 21, 22]. This is also supported by the fact that Class IIa HDACs possess an extended N-terminal domain that interacts with transcription factors, such as the myocyte enhancer factor (MEF)2 family. Studies have shown that the MEF2 transcription factor is involved in trophoblast invasion and differentiation in humans [23], while class IIa HDACs are also associated with cellular differentiation and development in several tissue types [21, 24]. Cell differentiation and development are important mechanisms seen during the secretory phase in the endometrium.

Recent studies have shown the involvement of class IIa HDACs (4, 5, 7, and 9) in regulating factors such as Hypoxia-inducible factor -1 alpha (HIF1α), implicated in human endometrial function [25, 26]. However, there is a lack of research showing their direct role in human endometrium, their expression levels, and the effects of hormone regulation.

This study aims to characterize the gene and protein expression of class IIa HDACs in endometrial tissue biopsies during different menstrual cycle stages. Further, the study also assesses the expression levels of HDACs in response to the steroid hormones and HDAC inhibitor TSA in HESCs.

Materials And Methods

Tissue Culture

The endometrial tissue biopsies were collected from premenopausal women, with the exclusion of samples from women with known endometrial pathologies or undergoing hormonal contraception within three months of sample collection. Ethical approval for the study was obtained from Northern X Ethics Committee (NTX/08/02/008). The stages were evaluated by a senior histopathologist using histological techniques.

Cell culture

Human endometrial stromal cells (HESCs) were used to study the effect of steroid hormones in class IIa HDAC expression [27]. The cells were cultured in Gibco Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1:1) phenol-free (Thermo Fisher Scientific). It was supplemented with 10% Charcoal stripped Fetal Bovine Serum (CS-FBS) and 1% antibiotic Penicillin-streptomycin (Thermo Fisher Scientific).

Steroid hormone treatment

2.5X 105 cells were plated in 6 well plates and incubated in humidified cell culture incubators at 37°C and 5% CO2 until 70% confluent. Each cell line was treated with β- estradiol (E2; 0.01μM) for 24 hours and subsequently with β- estradiol and progesterone (P4; 1μM) for 24, 48, and 72 hours. In addition, cells were also exposed to 1μM Trichostatin A (TSA) (Sigma-Aldrich, USA). All treatments were conducted in triplicates and included and untreated control which was prepared using ethanol and DMSO (Sigma-Aldrich). A treatments were prepared in analytical grade ethanol and DMSO using commercially available powdered concentrates (Sigma -Aldrich). The final concentrations were prepared in culture media and stored at -80º C.

RNA extraction, reverse transcription, and real-time qRT-PCR

RNA was extracted from both cells and tissue samples using Trizol® LS reagent (Life Technologies) according to manufacturers' instructions. 1ml Trizol® LS reagent was added to 50-100mg powdered frozen tissue samples or per well for cell cultures. Following the instruction manual, chloroform was added and centrifuged for 15 mins (12000xg) at 4°C leaving and aqueous phase. Isopropanol was added to the aqueous phase and following a 20 minute incubation on ice, was centrifuged using the same conditions. The RNA pellet was obtained, washed in 70% ethanol and centrifuged for 10minutes , twice. The pellet was air dried at room temperature and suspended in DEPC treated water and the concentration of samples was determined using the NanoPhotometer® N60 (Implen), while RNA integrity was assessed using Agilent 2100 bioanalyzer (Agilent Technologies). Samples with RNA integrity of seven and above were used for further analysis.

DNAse treatment was performed on 2ug RNA samples for 15 mins using DNase I Amplification Grade kit (Thermo Fisher Scientific). Using the manufacturer's protocol, a total quantity was made up to 10ul using

DEPC-water. 1ul of EDTA was added and the samples underwent a 10 minute incubation at 65°C. Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific) according to manufactures' instructions. Each tube comprised of- reverse transcriptase buffer, dNTP Mix, random primers, and Multiscribe Reverse transcriptase and made up to 20µLwith nuclease-free water. The reaction was run in BioRad DNA engine Peltier thermal cycler (BioRad Laboratories) at 25° C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and then held at 4°C until stored at -20°C.

RNA expression levels were quantified using qRT-PCR performed on QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific). The primer sequences and protocol (Table 1) were obtained from Dr. Sheryl Munro and perfromed under the following conditions: [6].

Activation at 95°C for 2 minutes.

1. 45 cycles of amplification.
2. Denaturation at 95°C for 15 seconds.
3. Annealing/extension at 60°C for 30 seconds.

Table 1 Primer sequence used for real-time PCR

Primers	Forward 5´-3´	Reverse 3´-5´	Amplicon size (bp)	Source
HDAC4	CCTCTACACATCGCCATCCT	GGCTGCTCCAGTAAGACCAT	237	[6]
HDAC5	GTAGCCATCACCGCAAAACT	GTCCTCCACCAACCTCTTCA	199	[6]
HDAC6	TATCTGCCCCAGTACCTTCG	GGACATCCCAATCCACAATC	242	[6]
HDAC7	GGCTGCTTTCAGGATAGTCG	TTCATCAGTTGCTGCGTCAT	162	[6]
YWHAZ	CCGTTACTTGGCTGAGGTTG	CAGGCTTTCTCTGGGGAGTT	189	[6]
RPLO	AGAAACTGCTGCCTCATATCCG	CCCCTGGAGATTTTAGTGGTGA	223	[6]
RPL13A	GCCCTACGACAAGAAAAAGCG	TACTTCCAGCCAACCTCGTGA	117	[6]

Protein extraction and Western blot

Frozen tissue samples were powdered using a sterile mortar and pestle, and protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific). According to the manufacturers protocol, 20µL Halt™ Protease Inhibitor Cocktail (EDTA-Free (100X)) (Thermo Fisher Scientific) was added per 1mL of RIPA lysis buffer. ~20µL of RIPA lysis buffer and Halt™ solution was added per microgram of tissue and samples were lysed using a tissue lyser at 30 frequency for 10 minutes. The cells were washed with ice-

cold PBS (1mL) and homogenized using ~400ul of RIPA and Halt™ mix in a sonicator. The tissue and cell lysates were incubated at 4°C on an orbital shaker for 2 hours and 30 minutes, respectively. Following which the samples were centrifuged at 4°C at 12000 rpm for 35 minutes to collect the supernatant protein. The concentration of samples was determined using Direct Detect® Infrared Spectrometer (MilliporeSigma).

10µg protein samples were run on Nu-Page 4-12% Bis-tris 15 lane mini gels according to the manufacturer's protocol. The proteins were transferred to Immobilon-FL PVDF Membrane (MilliporeSigma) for 2hrs at 30V. Membranes were stained with Revert™ 700 Total Protein Stain (LI-COR Biosciences) for 5 minutes using the single color western blot protocol and washed twice with Revert™ 700 Wash Solution (LI-COR Biosciences) and finally with MiliQ water. The membrane was dried and then visualized in the 700 nm channel with an Odyssey® imaging system, Classic Fc (LI-COR Biosciences). Following total protein imaging, the membranes were washed in 100% methanol and rinsed with MiliQ water. Membranes were blocked using Intercept Tris-buffered saline (TBS) Blocking Buffer (LI-COR Biosciences) for 1 hour at room temperature. Following blocking, the membranes were incubated in primary antibody overnight at 4°C, washed four times with 1X TBST in 5 minute intervals, and incubated in secondary antibody for 1 hour at room temperature on a rocking platform. The membranes were then washed again with 1X TBST four times in 5 minute intervals and rinsed with 1X TBS before imaging. Membranes were protected from light at all times following secondary antibody incubation and were imaged for 10 minutes at 800nm channel using Odyssey® imaging system, (LI-COR Biosciences) .

Target	Antibody	Supplier	Species	Band Size	Concentration
HDAC4	Ab32534	Abcam	Rabbit	119 kDa	1:5000
HDAC5	Ab55403	Abcam	Rabbit	122 kDa	1:500
HDAC7	Ab53101	Abcam	Rabbit	103 kDa	1:500
HDAC9	Ab109446	Abcam	Rabbit	111 kDa	1:15000

Antibody	Supplier	Species	Concentration
IRDye® 800CW Donkey anti-Rabbit IgG	LI-COR	Donkey	1:15000

Statistical analysis

The comparative CT method (ΔΔCT method) was used to analyze gene expression. Three housekeeping genes, YWHAZ, RPLO and RPL13a were used to normalize the amount of gene expression relative to the endometrial control sample. The data were analyzed using GraphPad Prism 8.2.1. The mRNA data during

the menstrual cycle was analysed using one-way ANOVA, and an unpaired t-test was done to compare each cycle stage. The normalized protein signal was plotted against the cycle stage for each sample and analysed using GraphPad Prism version 8.0. A one-way nonparametric ANOVA test was performed to determine the statistical significance for tissue protein expression. For the hormonal regulation data, the effect of treatment on the fold change in the protein expression was analysed using paired t-test ($P < 0.05$ was considered statistically significant).

Results

HDAC class IIa gene expression throughout the menstrual cycle in the endometrium

Temporal gene expression changes were observed in class IIa HDACs throughout the menstrual cycle (Fig 1). There were no significant changes in *HDAC4* mRNA expression throughout the menstrual cycle. mRNA expression of *HDAC 5* ($p \leq 0.001$) and *HDAC 7* ($p \leq 0.05$) were significantly upregulated during the early secretory phase compared to the proliferative phase, while expression of *HDAC 5* ($p \leq 0.0001$), *HDAC 7* ($p \leq 0.05$), and *HDAC 9* ($p \leq 0.01$) were significantly downregulated during late secretory phase as compared to early secretory phase (Fig 1). Furthermore, expression levels of *HDAC 5* ($p \leq 0.01$) and *HDAC 9* ($p \leq 0.05$) both were upregulated during the mid-secretory phase compared to late secretory and proliferative phases, respectively (Fig 1 (b) and (d)). *HDAC 5* ($p \leq 0.05$) expressionon was significantly downregulated during the late secretory phase as compared to proliferative phase (Fig 1).

HDAC protein expression in endometrium during the menstrual cycle

Both HDAC 5 ($p \leq 0.05$) and HDAC 9 ($p \leq 0.01$) protein expressions were significantly upregulated during the secretory phase compared to the proliferative phase (Fig 2 (b) and (d)), while no significant differences were observed in HDAC4 and HDAC7 protein expression (Fig (a) and (c)).

Changes in protein expression of HDAC classIIa in response to treatment with steroid hormones and TSA in HESCs, *in vitro*

There were little to no expressions of Class IIa HDAC mRNA in HESCs. However, significant changes in protein expression levels were observed in HESCs (Fig 3) in response to ovarian steroid hormones and a combination of steroid hormones and HDAC inhibitor TSA.

The HDAC4 protein expression was significantly downregulated in response to 24hrs of estrogen alone ($p \leq 0.05$) compared to control, while the expression levels were significantly upregulated after 48hrs of

treatment with combined estrogen-progesterone ($p \leq 0.05$) (Fig 3 (a)).

HDAC5 expression levels in HESCs treated with steroid hormones showed no significant changes throughout the treatment. . Whereas, the addition of TSA resulted in a significant down-regulation of HDAC5 protein expression after 72 hours. ($p \leq 0.05$) (Fig 3 (b)).

HDAC7 protein expression was significantly upregulated after 24hrs of treatment with estrogen and progesterone ($p \leq 0.05$). In contrast, the addition of TSA inhibited HDAC7 protein expression with significant downregulation after treatment with combined TSA and estrogen for 24 hrs($p \leq 0.0001$), 24hrs combined TSA and estrogen-progesterone ($p \leq 0.001$), and 48hrs combined TSA and estrogen-progesterone ($p \leq 0.05$) (Fig 3(c)).

Two bands were observed in HDAC9 (Fig 3 (d and e)), one at 110kda (HDAC9a) and another at 60kda (HDAC9b). A significant increase in expression of HDAC9a (110kda) was observed after 48hrs of treatment with estrogen and progesterone ($p \leq 0.05$), while its expression was significantly reduced after 72 hrs of treatment with estrogen and progesterone ($p \leq 0.01$) as compared to control. The expression level of HDAC9a was markedly decreased after treatment with a combination of TSA and estrogen for 24 hrs ($p \leq 0.01$) (Fig 3(d)). Similarly, HDAC9b (60kda) protein expression levels were significantly downregulated after treatment with the combination of TSA and estrogen-progesterone for 48hrs ($p \leq 0.01$) (Fig 3(e)).

Discussion

This study aimed to characterise the expression and regulation of class IIa HDACs in cyclic human endometrium and endometrial stromal cells, *in vitro*. Our results imply a cyclic expression pattern of Class IIa HDACs in human endometrial tissues, with upregulation during the early secretory and mid secretory phase. Furthermore, hormonal treatment of stromal cells showed significant changes in protein expression levels of class IIa HDACs in response to different combinations of steroid hormone treatments, with an increase in expression observed after treatment with combined estrogen and progesterone for 24 hrs in HDAC7 and 48 hrs in HDACs 4 and 9a. The treatment of HESCs with TSA combined with steroid hormones also demonstrated a significant inhibitory effect on HDACs 5, 7, and 9 expressions.

Post menstruation, the endometrial tissue lining starts to regenerate and proliferate, preparing for the next cycle. Increasing evidence suggests that Class 1 HDACs play a role in cell proliferation, and overexpression of HDACs 1, 2, and 3 proteins in cells have been implicated in endometrial cancer [28-31]. There are no studies on class IIa HDACs in the endometrium. However, other studies on class IIa HDACs suggest that they may be involved in tissue-specific development and differentiation [32-34]. HDAC5 is seen to be downregulated in several cancers, including prostate and breast cancer. CDK4/6 inhibitors inhibit CDK functions and thus induce cell cycle arrest and are being studied as an endometrial cancer treatment [35]. It is observed that HDAC5 deficient cells are linked to CDK4/6 inhibitor resistance in breast cancer cells [36], leading to cell proliferation. Our endometrial tissue mRNA expression data shows class

IIa HDAC 5 downregulated during the proliferative stage compared to the early secretory phase suggesting its possible negative regulation of proliferation.

In the endometrium, the secretory phase is marked by decidualization, which is the differentiation of human endometrial stromal cells in preparation of trophoblast invasion and subsequently implantation [37, 38]. It is a complex mechanism regulated by several factors [39]. An increase in H3K27ac is observed in HESCs during Decidualization, acting as an enhancer of insulin-like growth factor binding protein-1 (IGFBP-1), a decidualization marker [40]. In addition, the decidual-like morphology is induced by the action of progesterone on estrogen primed endometrium in combination with cyclic adenosine monophosphate (cAMP) [2, 41, 42]. Previous studies have reported the involvement of class IIa HDACs in cAMP signaling in various cell types [43], suggesting that a similar correlation could be involved in endometrial cells as well.

TSA has been observed to inhibit HDACs and enhance decidualization [44]. A study on endometrial stromal cells showed that TSA inhibits HDACs and subsequently enhances expression of decidualization markers insulin-like growth factor binding protein-1 (IGFBP-1) and prolactin [44]. TSA-treated HESCs inhibit HDAC and promote histone acetylation at the promoters of TIMP-1 and TIMP-3 by increasing the transcription of these genes [45]. Alternatively, another study showed that inhibition of HDAC5 by TSA decreases cAMP-induced expression of inducible nitric oxide synthase (iNOS) during HESC decidualization, demonstrating a role for HDAC5 in decidualization[46]. We observed an overall downregulation in class IIa HDACs 5, 7, 9a, and 9b protein expression in HESCs treated with combined TSA and steroid hormones. Thus, demonstrating that TSA mitigates the effect of steroid hormones in HESCs

During the late secretory phase, falling progesterone levels trigger an inflammatory response, marked by chemo- and cytokines production by decidualized stromal cells [37, 47, 48]. Class IIa HDACs, especially HDAC 4, have been shown to induce inflammatory responses [49-51]. Class IIa HDACs regulate cytokine expressionn, such as HDAC4 regulates IL-10 expression [52], while HDAC5 binds to IL-8 promoter resulting in inhibition of IL-8 expression [51, 53]. IL-2 expression is inhibited by HDAC7 and 9 interaction with FOXP3 and TIP60 [51, 54]. Class IIa HDACs 4 and 7 have been involved in regulating a crucial pro-inflammatory transcription factor Hypoxia-inducible factor (HIF)-1α, which is essential for vascularization in the premenstrual phase [50, 55, 56]. Significant downregulation was observed in *HDACs 5, 7,* and *9* during the late secretory phase in the endometrial cycle, while there was no significant change in *HDAC4* expression throughout. Class IIa HDACs could be involved in inflammatory responses in the endometrium.

Our data suggest that the class IIa HDACs might be involved more prominently in regulating gene expression during the secretory phase due to their upregulation during the early and mid-secretory phases. Similarly, in steroid hormone-treated HESCs, a significant increase in class IIa HDACs was observed upon the addition of progesterone which is known to induce decidualization in HESCs [41, 42]. Decidual HESCs make the significant component of the human decidua [38]. This study focused on the

HESC cell line to observe class IIa importance in secretory changes. Besides stromal cells, human decidua also comprises glandular cells, immune cells, and blood and lymph vessels [57]. Further studies on class IIa expression in immune cells and epithelial cell lines would better help us understand their menstrual cycle function. The mRNA expression levels of class IIa HDACs in HESCs were too low to be analyzed, but significant protein expression was observed, which could be due to rapid mRNA turnover [58, 59].

Two bands were observed for HDAC9 (9a and 9b). A similar presence of two isoforms for HDAC9 has been observed previously in other tissues and cells [60, 61]. HDAC9 has multiple alternatively spliced isoforms. In breast cancer cells, two isoforms have been observed one is a full-length HDAC9 with low expression levels and a truncated version that lacks the c-terminal deacetylase domain and is highly expressed in breast cancer cells [61]. Understanding the structural and functional differences in the two observed isoforms of HDAC9 in endometrial cells will give us a better understanding of their function in the endometrium. Further investigation is required to verify if there are any similarities between isoforms observed in this study and found in other tissues.

Class IIa HDACs have the unique ability to shuttle between nucleus and cytoplasm, resulting from post-translational modifications (PTMs) [18]. Other than regulation of subcellular localization, class IIa activities are also regulated by other PTMs, such as ubiquitin-dependent degradation [62]. In addition, they function as complexes by associating with other HDACs such as HDAC3 [63]. There is strong evidence of crosstalk between HDACs and other epigenetic factors in regulating cancer tumorigenesis [22, 54, 64].

Histone acetylation and HDACs have been involved in endometrial pathologies such as endometrial carcinomas and endometriosis [65, 66]. This study suggests the involvement of class IIa HDACs in the menstrual cycle regulation, most prominently in the differentiation of endometrium.

Declarations

Declaration of interest

The authors declare that no conflict of interest could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

PG: Overal research, data collection, analysis, writing the paper and designing the Figures. VM: Helped in deciding the flow of the research paper, editing and proof reading. AP: Overall supervision, conception and editing the article. The author(s) read and approved the final manuscript.

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Competing Interests

The authors declare that no conflict of interest could be perceived as prejudicing the impartiality of the research reported.

Non-Financial Interests

None

Ethics approval

Ethical approval for the study was obtained from Northern X Ethics Committee (NTX/08/02/008).

Consent to participate

The endometrial tissue biopsies were collected from premenopausal women after informed consent. The exclusion criteria included women with known endometrial pathologies or undergoing hormonal contraception within three months of sample collection

Consent for publication

Patients signed informed consent regarding publishing their data

Availability of data and material

The data will be made available upon request

Code availability (software application or custom code)

NA

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Figures

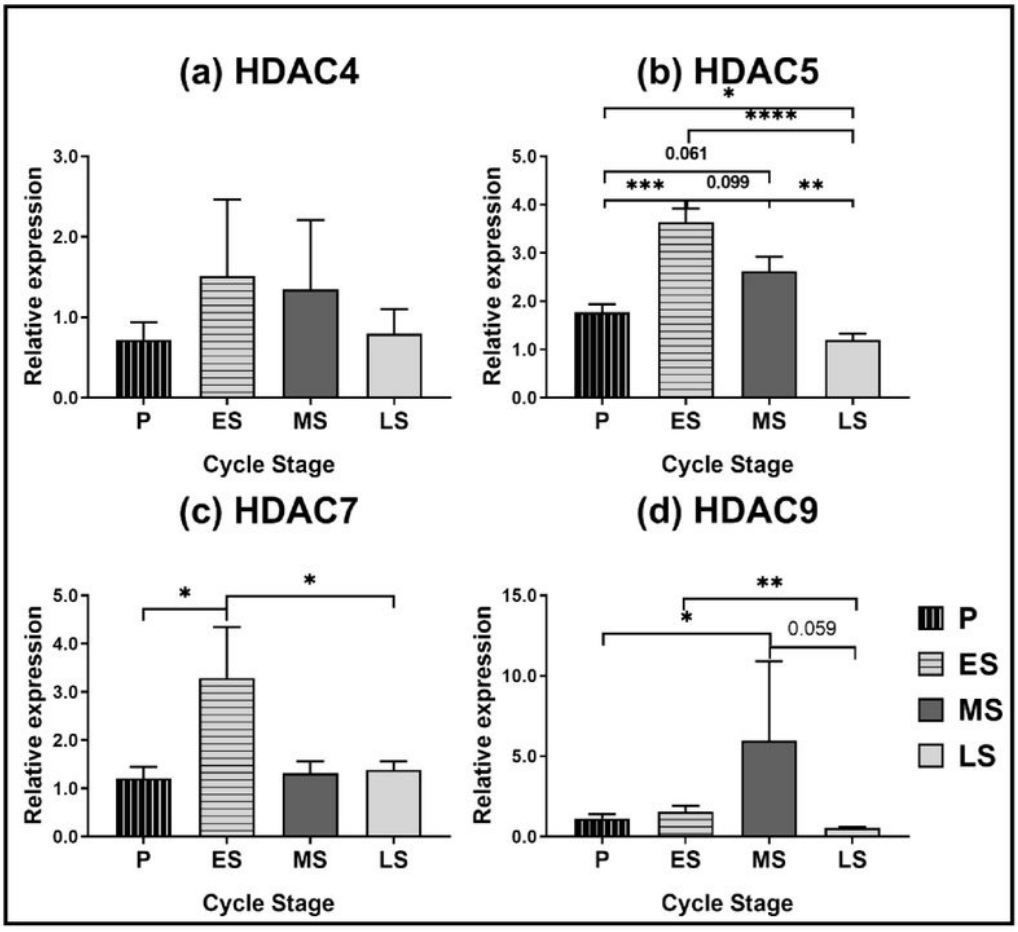

Figure 1

Relative mRNA expression of class IIa HDACs during menstrual cycle

Real time qPCR results for class IIa HDACs in human endometrium during the menstrual cycle stages: Proliferative (P) (n=10), Early Secretory (ES) (n=3), Mid Secretory (MS) (n=2) and Late Secretory (LS) (n=6). The Y-axis shows the relative mRNA expression normalized against three housekeeping genes

YWHAZ, RPLO and RPL13A represented as Mean±SEM. The X-axis shows the different menstrual cycle stages. Statistical significance determined using one-way ANOVA followed by unpaired t test and represented as *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001

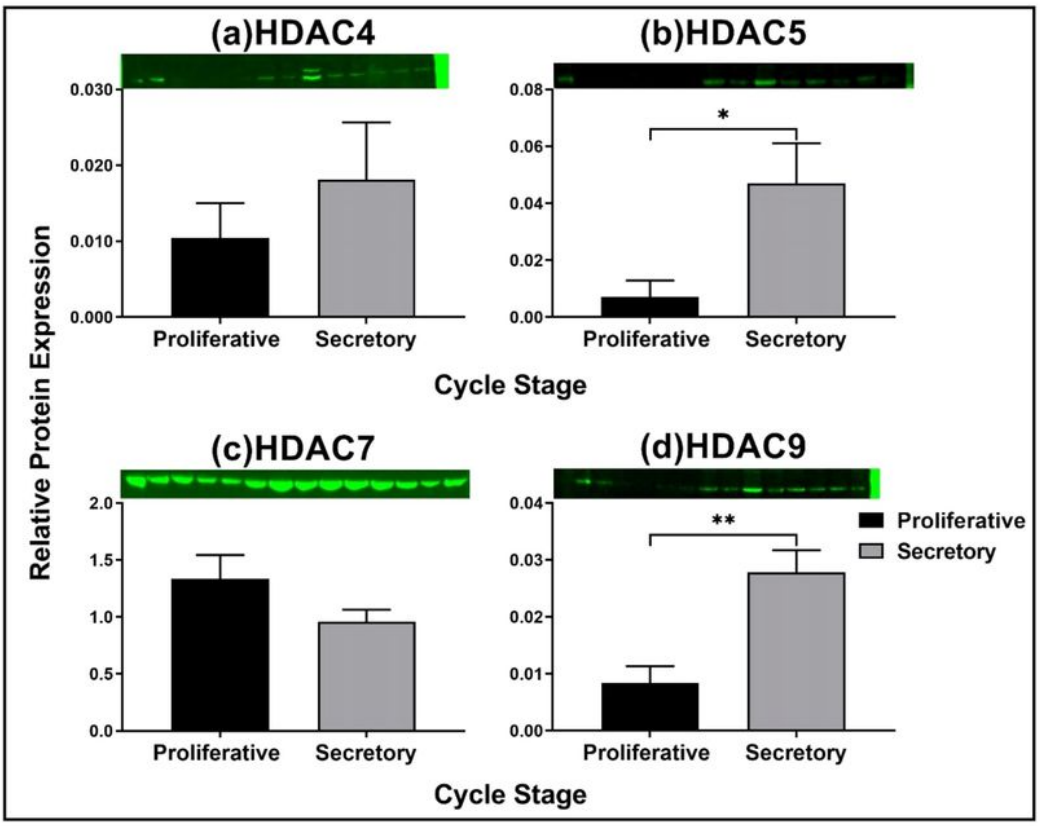

Figure 2

Changes in class IIa HDAC protein expression between Proliferative and Secretory phases

Relative protein expression levels normalized against total protein stain were plotted as Mean ± SEM against two cycle stages. Statistical significance is given as *p≤0.05 and **p ≤0.01. The fluorescent bands indicate from left to right Proliferative (n=7) and Secretory (n=7)

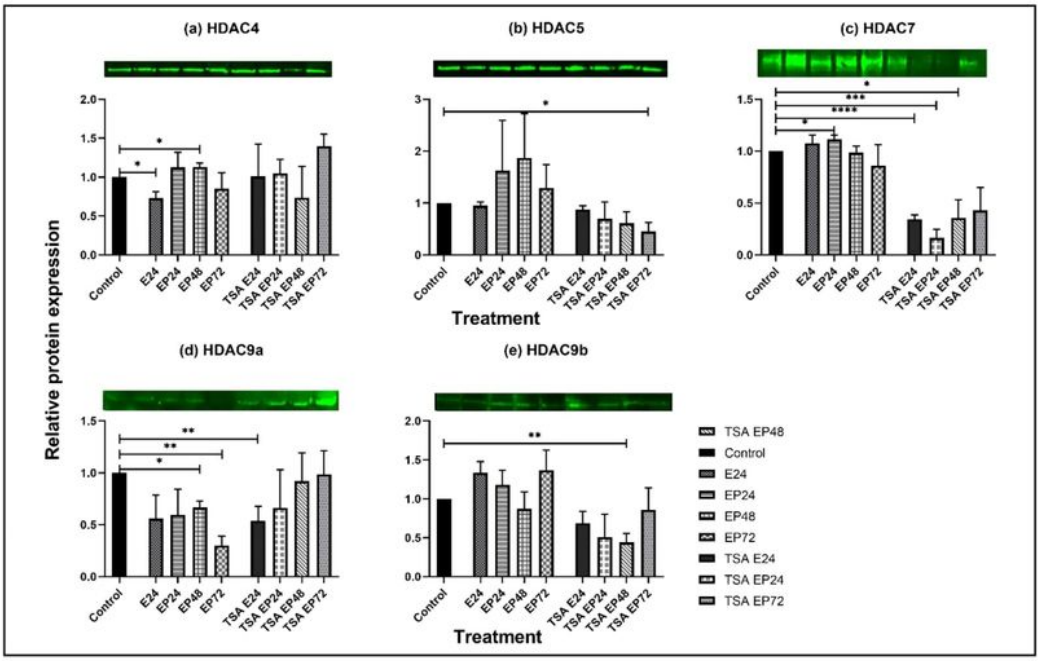

Figure 3

Changes in class IIa HDAC protein expression in HESC cells when treated with steroid hormones and TSA

Relative protein expression levels normalized against total protein stain and fold change was plotted as Mean±SEM against different treatment groups: Control; E24, 0.01μM Estrogen for 24hrs; EP24, combined 0.01μM Estrogen and 1 μM Progesterone for 24hrs; EP48, combined 0.01μM Estrogen and 1 μM Progesterone for 48hrs; EP72, combined 0.01μM Estrogen and 1 μM Progesterone for 72hrs. For each treatment group cells were also exposed to 1μM Trichostatin A (TSA). Statistical significance is given as *p≤0.05, **p ≤0.01, ***p ≤0.001 and ****P<0.0001