Expression and regulation of histone deacetylases in human endometrium during the menstrual cycle

Palak Gujral

A thesis submitted in partial fulfilment of the requirements for the degree of

Master of Biomedical Science,

The Liggins Institute, The University of Auckland, 2020.

Abstract

Histone acetylation is a critical epigenetic modification that changes chromatin architecture and regulates gene expression by opening or closing the chromatin structure. It plays an essential role in cell cycle progression and differentiation. The human endometrium goes through cycles of proliferation, differentiation, and degradation each month requiring strict epigenetic regulation for the proper functioning of the endometrium. Histone acetylation is generally associated with gene activation. It is regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs), which include sirtuins (SIRTs). Previous study from our lab has demonstrated that global histone acetylation changes in the endometrium correlate with the expected transcriptional activity during the menstrual cycle. However, the expression and regulation of HDACs in the endometrium have not been elucidated. The aim of this master's project is to characterize the gene and protein expressions, and hormonal regulation of HDACs in human endometrium during the menstrual cycle. Normal endometrial tissues were obtained from cycling pre-menopausal women. Gene and protein expression patterns for the 18 HDACs were determined across the menstrual cycle stages. The analysis established the profile for HDAC expression in human endometrium during the menstrual cycle. In addition, effects of steroid hormone and HDAC inhibitor TSA were studied in three endometrial cell lines. The AN3 cells were used as a model for non-receptive endometrium epithelium, while RL95- 2 cells were used as a model for receptive epithelium. Human endometrial stromal cells (HESCs) were used as stromal cell models.

The mRNA data showed that many of the HDACs were upregulated during the early secretory and mid secretory stages. The protein expression profiles matched the gene expression patterns in the endometrial tissues. The steroid hormone treatment showed that the gene expression of HDACs vary widely in the different cell lines in response to hormones. The effects of TSA on different HDACs also produced cell line specific effects. The results imply that there is temporal regulation of HDACs in endometrium during the menstrual cycle and is influenced by steroid hormones. Further functional studies to investigate the roles of specific HDACs will lead to better understanding of the regulation of histone acetylation in endometrium.

Acknowledgments

I would like to express my deep and sincere gratitude to my supervisor Dr. Anna P. Ponnampalam, whose unending guidance and support helped me throughout this project. I can't thank you enough for the invaluable knowledge you have provided me.

A special thanks to my co-supervisor Dr. Jo Kate Perry, for being there whenever I had doubts about my future.

I am deeply grateful for the help and support provided by Ms. Hui Hui Phua, for teaching me the techniques and providing her invaluable tips to get better results.

I would like to thank Ms. Vishakha Mahajan for always being there for me and answering all my silly questions.

My thanks also go to the administrative staff Mrs. Jean Leonard for help throughout my project.

I would also like to thank Dr. Sue R McGlashan without whose help I would not have been here.

I express my sincere thanks to my husband for always encouraging me and giving wings to my dreams. And a special thanks to my parents and in-laws for supporting me through this journey.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AN3	Acanthosis Nigricans 3rd Attempt
BRAP2	Brca1 Associated Protein 2
CBHA	M-Carboxycinnamic Acid Bishydroxamide
COL1	Collagenase
DMSO	Dimethyl Sulfoxide
DNMT	DNA Methyltransferases
EC	Endometrial Carcinomas
ECM	Extracellular Matrix
eMSC	Endometrial Mscs
EP	Estradiol And Progestrone
ER	Estrogen Receptors
ES	Early Secretory
ESC	Endometrial Stromal Cells
ESS	Endometrial Stromal Sarcoma
FBS	Fetal Bovine Serum
FSH	Follicular Stimulating Hormones
GNAT	Gcn5-Related N-Acetyltransferase
HAT	Histone Acetyl Transferases
HBO1	Human Acetylase Binding To ORC1
HDAC	Histone Deacetylases
HESC	Human Endometrial Stromal Cells
HMOF	Human Males-Absent-On-The-First
HPG	Hypothalamic-Pituitary-Gonadal
HPO	Hypothalamic-Pituitary-Ovarian
HPT	Hypothalamic-Pituitary-Testis
IGFBP	Insulin-Like Growth Factor Binding Protein-1
iPSC	Induced Pluripotent Stem Cells

KO	Knockout
MMP	Matrix Metalloproteinases
MORF	MOZ-Related Factor
MOZ	Monocytic Leukemic Zinc Finger
MS	Mid Secretory
MSC	Mesenchymal Stem Cells
MW	Molecular Weight
NAD	Nicotinamide-Adenine Dinucleotide
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
PCAF	P300/CBP-Associated Factor
PCR	Polymerase Chain Reaction
PR	Progesterone Receptor
PTM	Post-Translational Modifications
SAHA	Suberoylanilide Hydroxamic Acid
SIRT	Sirtuins
TBS	Tris-Buffered Saline
TIMP	Tissue Inhibitors Of Mmps
TSA	Trichostatin A
uNK	Uterine Natural Killer Cells
USP	Ubiquitin-Specific Proteases
VEGF	Vascular Endothelial Growth Factor
VPA	Valproic Acid

Publications

Part of the literature review has been published as the following review article (1):

Histone acetylation and the role of histone deacetylases in normal cyclic endometrium

Palak Gujral, Vishakha Mahajan, Abbey C. Lissaman & Anna P. Ponnampalam

1 Introduction

The human uterus, also is known as the womb, is a hormone-responsive reproductive organ situated in the female pelvis. It provides a nourishing and protective environment to the foetus throughout gestation (2). The innermost lining of the uterine cavity is called the endometrium. The endometrium goes through cyclic changes every month called the uterine cycle or menstrual cycle. The events of the uterine cycle are parallel to those of the ovarian cycle, which is in turn, regulated by the pituitary glands (3). The cycles of proliferation, differentiation, menstruation, and regeneration are highly regulated processes.

1.1 The Uterus

The uterus is a pear-shaped female secondary sex organ. Its primary functions are to maintain and transport female sex gametes, “ova,” facilitate fertilization, and maintain pregnancy. It has three major parts: the fundus is the top part which connects fallopian tubes to the body; the body is the uterine cavity where implantation takes place; There is a narrow constriction between the body and the cervix of the uterus called the isthmus (4), which leads to cervix the lower part of the uterus which opens to vagina (2, 5, 6)

The uterus is made up of three tissue layers (2):

- Endometrium: This is the innermost mucous lining of the uterus; it sheds every month during menstrual bleeding.
- Myometrium: This layer lies beneath the endometrium and is made up of smooth muscle.

- Peritoneum: It is the outermost lining and is continuous with the abdominal peritoneum.

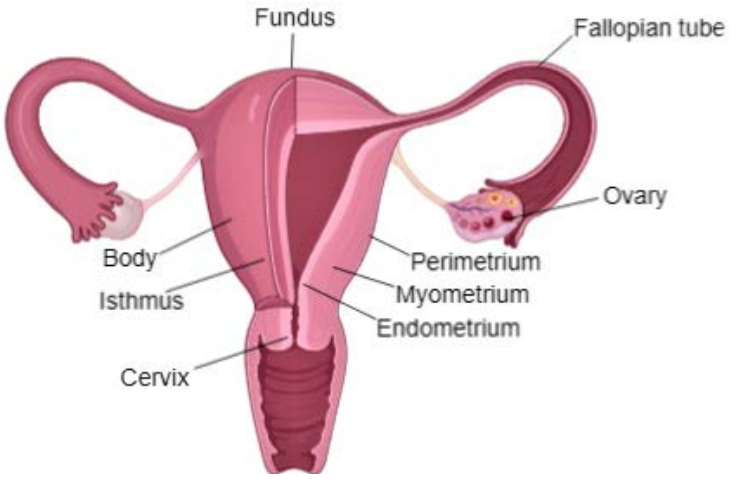

Figure 1: Uterus *Created using BioRender.com

1.2 The Endometrium

The human endometrium is the innermost lining of the uterus. It originates from the urogenital ridge in early development (7). Its fundamental function is to provide an immunoprivileged site for embryo implantation and a nurturing environment for fetal development (8).

The endometrium is characterized by distinct cell types: luminal epithelium, glandular epithelium, and stromal cells (7-10). It is made up of three layers: the stratum compactum and stratum spongiosum make up the stratum functionalis, which breaks up during menstruation and is subject to hormonal changes; the stratum basalis is nearest to the myometrium and contains the cells from which the functionalis layer regenerates after menstruation (11, 12). The functionalis layer goes through cyclic morphological and functional changes, called the menstrual cycle, under the influence of ovarian steroid hormones. These changes are divided into three phases: The proliferative phase, the secretory phase, and menstruation (8, 13). The Hypothalamic-Pituitary-Gonadal (HPG) Axis coordinates these endometrial phases.

1.2.1 Hypothalamic-Pituitary-Gonadal (HPG) Axis

Hypothalamic-Pituitary-Gonadal (HPG) Axis is the co-function of the hypothalamus, pituitary, and gonadal glands as a single entity. It is critical for human reproduction in both males and females (14). In females, it regulates the ovarian and menstrual cycle. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) which is responsible for puberty onset and hormone regulation in females. It stimulates the pituitary glands to secrete gonadotropins, luteinizing hormone (LH) and follicular stimulating hormones (FSH) (15). The gonadotropins stimulate the production of steroid hormones estrogen and progesterone in females, and testosterone in males (16). The HPG axis is called the Hypothalamic-Pituitary-Ovarian (HPO) Axis in females and Hypothalamic-Pituitary-Testis (HPT) Axis in males. The estrogen produced by ovaries acts in a negative feedback loop and inhibits the production of GnRH (17). The GnRH is produced in a pulsating manner from the hypothalamus; the male and female axes are different due to the presence of a gonadotropin surge in mid ovarian cycle in females, which triggers ovulation. This occurs due to high estrogen levels secreted from a dominant

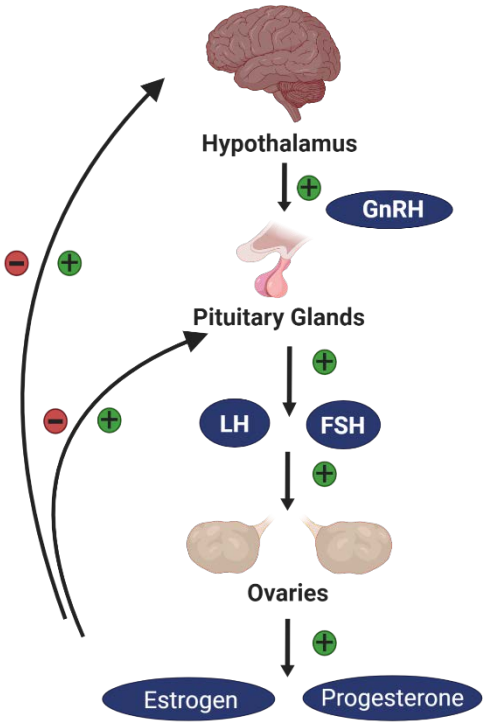

Figure 2: Hypothalamic-Pituitary-Gonadal (HPG) Axis in females
***Created using BioRender**

follicle (18). During the midcycle gonadotropin surge, estrogen acts in a positive feedback loop on the HPG-Axis and encourages the production of GnRH (17).

1.3 The Menstrual Cycle

Each month the endometrium goes through cycles of proliferation, differentiation, menstruation, and regeneration up to 400 times in a lifetime; this is called the menstrual cycle (19). Menarche, which is the start of the first menstrual period, generally occurs between 11 and 15 years of age, while menopause, which is the end of the last menstrual period in a woman's life, occurs between 45 and 55 years of age (20). The menstrual cycle starts on the first day of menstrual bleeding and will last for an average of 28 days (21).

The endometrial changes during the menstrual cycle are controlled by, and synchronized with, the ovarian cycle events, involving the development of an ovarian follicle and production of steroid hormones (22). The ovarian cycle, controlled by pituitary hormones FSH and LH(23), is divided into three phases: the follicular phase, ovulation and luteal phase. The ovarian or gonadotropic hormones estrogen and progesterone (23) are secreted during these phases to regulate the uterine cycle, also known as the endometrial cycle.

The endometrial cycle is divided into three major phases: the proliferative phase, the secretory phase, and menstruation (Figure 3).

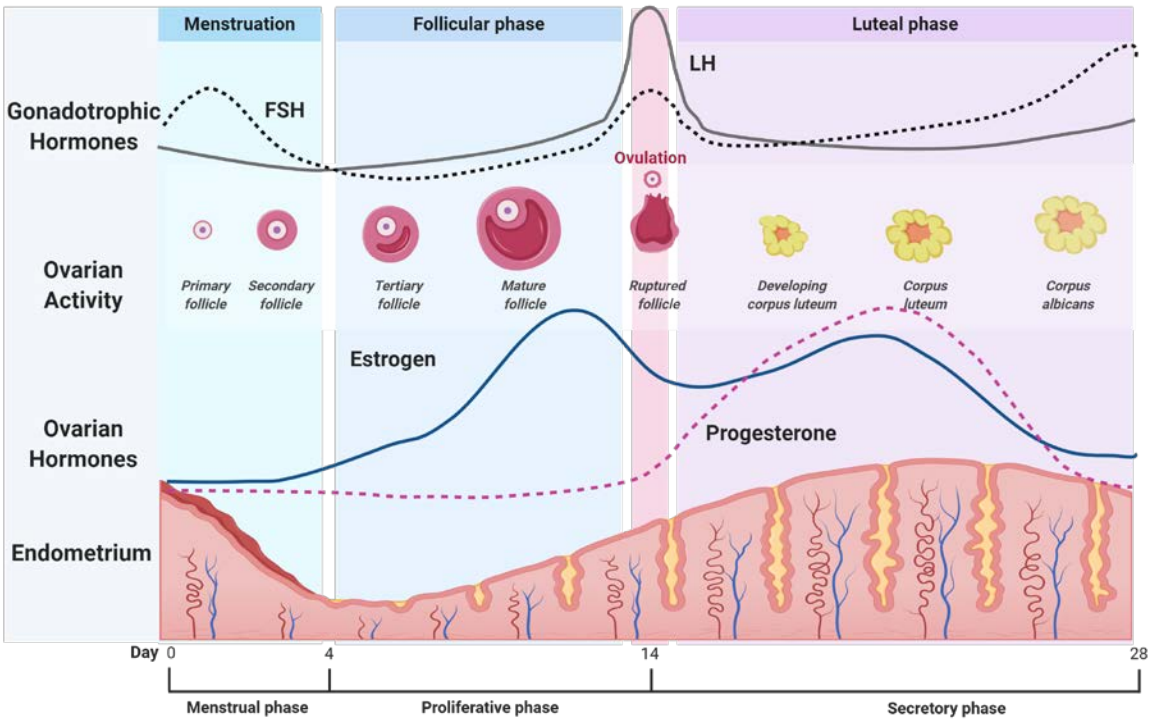

Figure 3: The ovarian cycle and uterine cycle events controlled by gonadotropic and steroidal hormones *Created using BioRender.com

1.3.1 Proliferative Phase

The proliferative phase starts from approximately day 5 of the menstrual cycle and ends on the 14th day, although it varies between individuals (24). The proliferative phase of the endometrium runs parallel to the ovarian follicular cycle, and it is regulated by estrogen secreted from developing ovarian follicle. The rising levels of estrogen help in restoring the functionalis layer of the endometrium (25), cell proliferation leading to the thickening of the endometrium, growth of endometrial glands, and emergence of spiral arteries. The blood-flow through the endometrial vessels increases throughout this phase, keeping pace with the tissue growth, and it reaches its peak 48 hrs before ovulation (3).

This phase is further subdivided into three stages: early proliferative, mid proliferative, and late proliferative. The early proliferative stage is characterized by a thin epithelium and narrow glands embedded in the stroma of cells. In general, the early proliferative stage starts from day

5 and ends on day 7 of the menstrual cycle. The endometrium is usually 2mm thick during this phase. The stroma is compact with spindle-shaped cells, with dense nuclei and minimal cytoplasm (26, 27). Days 8 to 10 of the menstrual cycle are usually considered the mid proliferative stage (26), characterised by columnar epithelium and longer glands. The epithelium increases in height, and a variable amount of stromal edema is seen during this phase (26, 27). Days 11 to 14 of the menstrual cycle marks the late proliferative stage (26). In the late proliferative stage, the glands continue to grow and become more convoluted [3], and pseudo stratification of columnar epithelial cells can be seen (26, 27).

1.3.2 Secretory phase

The secretory phase starts after ovulation when the endometrium undergoes differentiation. It lasts up to 14 days in most women [31] and occurs in response to the increased serum progesterone secreted by the corpus luteum [32]. Both estrogen and progesterone act together during this phase, facilitated by luteinizing hormone (LH). Glandular epithelial cells accumulate glycogen rich vacuoles and display large mitochondria with cristae and a considerable amount of endoplasmic reticulum during this phase [33]. The stromal proliferation slows down, and secretory transformations are seen in glands as they start to secrete more mucus [34].

The secretory phase is further subdivided into three stages: early secretory, mid secretory, and late secretory.

The early secretory stage generally dates post-ovulatory day 1-5, and is characterized by glands with abundant endoplasmic reticulum, accumulation of glycogen-rich vacuoles, and nuclei displacement [34]. The glandular epithelial cells and stromal cells develop nuclear heterochromatin (8). During this phase, there is an increase in stromal edema, which results in endometrial thickening (8, 28).

During the mid secretory phase, generally dated post-ovulatory days 6-8, increased coiling and lengthening of spiral arteries can be seen [35]. The endometrial glands are tortuous during this phase, and the secretory activity reaches its peak about six days after ovulation; this can be observed by the loss of vacuoles from the cytoplasm of the epithelial cells (8, 28).

Late secretory phase, generally dated post-ovulatory days 9-12, is characterized by predecidualized cells, which are stromal cells with increased size and volume, and spiral arterioles [8]. Some stromal cells develop into granular stromal cells, which have a dense kidney-shaped nucleus and granules in their cytoplasm (28). All the stromal cells have differentiated towards the end of this stage, and surface epithelial cells become cuboidal with rounded nuclei (28). The endometrium thickens and prepares itself for implantation. Any fluctuations in the duration of this phase can lead to infertile cycles in women [28].

1.3.2.1 Decidualization

Decidualization is the process of non-reversible morphological and functional differentiation of the human endometrial stromal cells in preparation for pregnancy (29, 30). It is initiated during the mid secretory phase at the presumed window of implantation, approximately six days after ovulation (8, 31, 32), in response to increased progesterone levels during the menstrual cycle (30). The decidual changes are crucial for embryo implantation and maintenance of pregnancy (33). Decidualization begins in the absence of conception in humans and therefore is a probable reason for menstruation (34). The decidualization process starts with the stromal cells surrounding the arteries present in the stratum functionalis (30), and with morphological changes occurring in the endometrial stromal cells (ESCs). It is followed by the transformation of uterine glands, the influx of uterine natural killer cells, and the development of blood vessels to support the developing fetus (35, 36). A build-up of glycogen and lipids is seen in decidualised stromal cells, giving them a bulky epithelial cell-like characterization in

contrast to their spindle-shaped appearance during the proliferative phase. Cells are enlarged, with a rounded nucleus and enlarged Golgi bodies and rough endoplasmic reticulum (37). The process is marked by the transformation of endometrial fibroblasts into secretory cells. Decidualized ESCs provide nutrition for the implanting blastocyst (38). Decidualized stromal cells are essential for the development of uterine trophoblastic, hematopoietic, and mesenchymal cells (39). The decidualization process also plays an integral part in maintaining the immune response during pregnancy, by facilitating the influx of leukocytes such as uterine Natural Killer cells (uNKs) (37).

1.3.2.2 Leukocyte Infiltration

Leukocyte infiltration is a massive influx of leukocytes that triggers an inflammatory reaction. The leukocyte numbers are low during the proliferative stage. During the late secretory phase, in the absence of pregnancy, the progesterone and estrogen levels in the uterus fall, which triggers the production of chemokines and cytokines by decidualized stromal cells (29, 40, 41). The leukocytes invade the tissues and become activated, producing enzymes such as matrix metalloproteinases (MMPs), which facilitate the degradation of the endometrium. Half of the premenstrual endometrial cell composition consists of inflammatory cells, which go through an array of events causing tissue degradation and menstruation (34). The CD45+ leukocyte composition has been demonstrated by *Starkey et al.* to be at peak levels just before menstrual bleeding commences (42). Uterine Natural Killer cells (uNKs) are the primary leukocytes involved in menstruation (43), accompanied by macrophages and granulocytes (34, 43).

Aside from menstruation, inflammation is a critical part of other endometrial stages like ovulation, implantation, and pregnancy as well. Implantation of the embryo can induce an immunological response characterised by leukocyte infiltration (43). 70% of the cells at the implantation site are uNKs, which control placental invasion (44, 45).

Leukocytes have both direct and indirect functions in menstruation. Some of their secretions are directly responsible for tissue degradation, while some activate enzymes secreted by other cells (46).

1.3.2.3 Uterine Natural Killer cells (uNK)

Uterine natural killer cells (uNKs) are present in small numbers during the proliferative and early secretory phases, and increase in number during the late secretory phase (45). They are the most abundant lymphocytes in the endometrium during early pregnancy (47). CD56++/CD16- are the uNKs found in human endometrium; they have a highly granulated appearance compared to CD56++/CD16- in blood (48). They have been associated with trophoblast invasion, vascular development, and angiogenesis. The primary function of uNK cells is the secretion of cytokines, growth factors and angiogenic factors (49, 50). They are known to produce metallomatrix proteases (MMPs), which are associated with the degradation of the endometrium during menstruation (49).

1.3.3 Menstruation

Menstruation is a highly controlled inflammatory process. It occurs in the absence of pregnancy and is due to the withdrawal of steroidal control of the endometrium and subsequent events leading to endometrial breakdown (40). The endometrium sheds itself at the end of every non-conception menstrual cycle. Menstruation in most women normally lasts up to 4-5 days (51). The first day of bleeding is considered to be the first day of the menstrual cycle (34). The average amount of blood lost during menstruation is about 30 to 40 ml, though it can be as low as 15 or up to 80 ml. 80% of the blood lost during menstruation occurs during the first 2 days (52). The endometrium goes through rapid degeneration and regeneration during this period (51), in response to the rapid fall in progesterone and estradiol-17β (estrogen steroid hormone) levels and degeneration of the corpus luteum. The endometrial stroma undergoes

decidualization during the secretory phase, even in the absence of an embryo (53). As decidualization is irreversible, the endometrium has to be shed and replaced to provide an opportunity for implantation every month. While shedding occurs, the surface is rapidly re-epithelialized (34). At the end of menstruation, the primordial follicles start developing, marking the starting of the proliferative stage (54).

1.4 Steroidal Control in the Endometrium

The female reproductive system includes the gonads (ovaries), which have an important endocrine function. The gonadotrophin releasing hormone (GnRH) from the hypothalamus stimulates the production of luteinizing hormone (LH) and follicular stimulating hormone (FSH) from the pituitary glands, which in turn regulate the production of steroidal hormones estrogen and progesterone from the ovaries (18).

The changes in the endometrium depend on the steroid hormones acting through specific down-stream mechanisms involving complex molecular signalling (8). The steroidal hormones estrogen and progesterone are major regulators of the menstrual cycle and are highly involved in the development of the endometrium (11). In fact, the drop in levels of steroidal hormones leads to the breakdown of the endometrium, resulting in menstruation (40).

1.4.1 Estrogen

Estrogen is a reproductive hormone secreted by the ovaries. It regulates the ovulation process in the menstrual cycle and promotes the thickening of the endometrial wall. Estrogen is also produced by the placenta in pregnant women. There are three types of naturally occurring estrogen: estrone (E1), estradiol (E2), and estriol (E3) (55). Estradiol (E2) is the primary form of estrogen in reproduction (56).

1.4.1.1 The Function of Estrogen in the Menstrual Cycle (18):

1. Increases size and height of epithelial cells, facilitating proliferation.
2. Acts in increasing blood flow to the oviducts, production of glycoprotein, and promotes the production of cilia throughout the oviduct.
3. Promotes thick mucus production in the isthmus.

1.4.2 Progesterone

Progesterone is a reproductive hormone produced by the corpus luteum. It prepares the endometrial lining for the reception of the fertilized egg.

1.4.2.1 The Function of Progesterone in the Menstrual Cycle (18):

1. Maintenance of the endometrium lining.
2. Promotes removal of cilia.
3. Decreases mucus production in the isthmus.

1.4.3 Steroid Receptors

Steroid hormones bind to steroid hormone receptors, initiating signal transduction, and the control of changes in gene expression (57, 58). In the endometrium, the steroidal response depends on the number of hormones available. Estrogen receptors (ER) and progesterone receptors (PR) are two nuclear receptors involved in endometrial regulation (8).

1.4.3.1 Estrogen Receptors

The endometrium is the primary target for estrogen and responds to the estrogen stimulation via estrogen receptor signalling (59-61). There are two kinds of estrogen receptors, ERα and ERβ, which belong to the steroid hormone superfamily of nuclear receptors (62). The estrogen effects in the uterus are primarily mediated by ERα which was first sequenced in the year 1986

(8, 63), while ERβ was discovered later in the year 1996 (64). The expression of ERα is consistent throughout the basal layer, but varies in the functional layer with expression at its peak during the proliferative stage and decreased during the secretory stage (65, 66). Studies in knockout mice have shown that ERα and ERβ deficiencies can lead to infertility in female mice (67). ERα is highly expressed in the epithelium, promoting the proliferation of epithelial cells through estradiol signalling. ERα and ERβ counteract each other. It has been found that ERβ modulates ERα-mediated transcription of genes in the uterus, and is responsible for the downregulation progesterone receptors (PR) in the luminal epithelium (67). Uterine growth and differentiation is regulated by estradiol-17β, known as E_2, which also acts on estrogen receptors (68).

ERβ is not shown to have a direct effect on the reproductive system, but it appears to regulate the response of ERα (69). The ERβ mRNA expression is very low throughout the menstrual cycle, while the protein is abundantly expressed, especially in epithelial cells (69, 70). Studies on ERβ in knockout mice have shown that it regulates the effects of ERα and maintains dormancy in the immature uterus (59, 71).

1.4.3.2 Progesterone Receptors

Progesterone is another female sex hormone, and its main function is the maintenance of endometrium. Progesterone receptors have two isoforms: PR-A and PR-B; which are both generated from a single gene. The PRs are a part of the ligand-activated transcription factor family and are comprised of a regulatory region, a DNA binding domain, and a ligand-binding domain (72). The PR-B receptor is comprised of an additional 164 amino acids at the N-terminus compared to PR-A (73).

Estrogen stimulates endometrial cell proliferation, while progesterone inhibits it. PR-A and PR-B have different functions (74, 75). PR-A is essential to mediate the decidualization of

stromal cells through the action of progesterone (76). Knockout studies have shown that PR-A is vital for the inhibition of proliferation stimulated by estrogen and PR-B signalling (77), while PR-B functions as an activator of progesterone target genes (78, 79). PR-A and PR-B both have elevated protein expression during the proliferative phase and reach their peak in the late proliferative phase (80). PR levels fall during the mid-secretory phase, allowing a change from regulation by ovarian steroids to paracrine regulation by stromal factors, while PR-A levels fall during the secretory phase (81). PR-B expression remains constant in the epithelial cells, corresponding to its involvement in glandular functions, however PR-A is continuously expressed throughout the menstrual cycle in stromal cells (82).

The progesterone response is regulated by the combined activity of PR-A and PR-B. Both isoforms are differentially expressed, and the maintenance of their ratio is vital to maintain the normal endometrium state (82). Disruption of the ratio of PR-A and PR-B can lead to many endometrial conditions such as endometriosis (79).

1.5 Epigenetics

As defined by C.T. Wu and J.R. Morris, epigenetics is the study of heritable changes in gene function without changes to the DNA sequence (83). These changes are reversible and include DNA methylation, histone modification, chromatin remodelling and regulation using non-coding RNAs like microRNAs. Cell differentiation, genomic imprinting, and X-chromosome inactivation are some of the cellular phenomenons that are epigenetically regulated (84). Epigenetic regulations are being studied as potential diagnostic and therapeutic methods for conditions such as cancer, endometriosis, chromosomal instabilities and neurodevelopmental disorders (85, 86).

1.5.1 DNA Methylation

DNA methylation is the most commonly studied epigenetic phenomenon in the human genome. The methylation of promoters results in repression of transcription, while gene methylation is linked to gene expression. DNA methylation patterns can be affected by external factors such as age, diet, parenting, and lifestyle (87). More than 98% of DNA methylation is observed at CpG dinucleotides in somatic cells (88). Changes in DNA methylation have been seen to affect immune responses and the extracellular matrix pathway in the endometrium (89). Inhibition of DNA methylation by 5-aza-2′-deoxycytidine has been involved in downregulating stromal cell proliferation and decidualization in the endometrium (90). DNA methylation marks are established by various DNA methyltransferases (DNMTs) The DNMTs are categorized into the following subcategories: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (88).

1.5.2 Histone Modifications

Approximately 2 meters of DNA is packaged inside a nucleus with the help of proteins called histones, forming a DNA-protein complex known as chromatin. The fundamental unit of chromatin is called the nucleosome, which consists of two sets of the four core histones: H2A, H2B, H3, and H4 (91). Histone H1 links the nucleosomes together to form a chromosome (92). Each of these nucleosomes are separated by linker DNA, resulting in a nucleosome array structured like beads on string, which further condenses to form a chromatin fiber. The globular domains of histones mediate histone–histone interactions, and amino acid terminals extend from the surface to control the folding and unfolding of the chromatin structure, aiding in the silencing and activating genes (93).

Histones have a protruding tail at the amino-terminal, which is subject to post-translational modifications (PTMs), such as acetylation, phosphorylation, lysine methylation, arginine methylation, deamination, ADP ribosylation, proline isomerization, β-N-acetylglucosamine,

ubiquitylation, and sumoylation (94). Histone modifications regulate chromatin structure; facilitate remodelling; and affect DNA processes such as replication, repair, and recombination (94). Most of these histone modifications are reversible, and provide scope for developing novel therapeutic methods.

1.5.3 Histone Acetylation

Histone acetylation is the addition of an acetyl group to lysine residues on the protruding histone tails (94, 95). It is the most common epigenetic marker and strongly affects gene expression (96). It affects various cellular functions like DNA recombination, cellular proliferation, cellular differentiation, apoptosis, and DNA repair (97). While histone acetylation is usually associated with transcriptional activation, histone deacetylation is associated with gene silencing (98). Histone acetylation patterns are maintained by two opposing groups of enzymes: the histone acetyltransferases (HATs) and histone deacetylases (HDACs) (99). HATs are responsible for the addition of an acetyl group, and HDACs remove them (94).

1.5.4 Histone Acetyltransferases (HATs)

HATs add an acetyl group to the ε-amino group of lysine using acetyl CoA as a cofactor, which neutralizes the positive charge on lysine, weakens the histone-DNA interaction, and makes genes accessible (94).

HATs are a diverse set of proteins. So far, about 30 HATs have been identified in humans. HATs are primarily classified into two classes based on their subcellular localization: Type A and Type B. Type A HATs are localized in the nucleus, while Type B is found in the cytoplasm. The Type A HATs function in transcription-related histone acetylation in chromatin, while

Type B HATs acetylate newly synthesized histones and influence the structure of the nucleosome (100, 101).

Type A HATs are further grouped into five families based on their catalytic domain (102, 103). The Gcn5-related N-acetyltransferase (GNAT) family includes P300/CBP-associated factor (PCAF), Gnc5, and ELP3; the MYST family includes Tip60, monocytic leukemic zinc finger (MOZ), MOZ-related factor (MORF), human acetylase binding to ORC1 (HBO1), and human males-absent-on-the-first (HMOF) (103, 104); the CBP/p300 family includes CBP and p300 (103, 105), and the transcription factor related family includes TAF1 and TIFIIIC90. Besides these, HATs also include many steroid receptor co-activators (103, 106, 107).

Deregulation of HATs is associated with cancer formation (108). CBP/p300 is essential for the transition from G1 to S stage in the cell cycle and can function as either a tumor suppressor or an oncogene depending on its localization (109, 110). Selective inhibition of p300 inhibits the cell cycle and induces apoptosis. It inhibits the response to DNA damage in melanoma cells (111). MYST family HATs have various roles in stem cell function and development (104). Because HATs are reversible regulators and are involved in cell cycle progression, they are being studied as targets for tumor growth management (112).

1.5.5 Histone Deacetylases (HDACs)

The name histone deacetylase is a misnomer in itself as HDACs are post transcriptional modulators which deacetylase both histone and also non-histone proteins, removing acetyl groups from lysine residuce (113). In humans, 18 HDACs have been identified and grouped into four classes based on their sequence homology to yeast (113, 114): 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). Class I, II, and IV HDACs are Zn^{2+} dependent enzymes and have similar functional mechanisms, whereas class III HDACs are nicotinamide-adenine dinucleotide

(NAD+) dependent deacetylases. HDACs have relatively low substrate specificity; one HDAC can act on multiple substrates, or multiple HDACs can act on the same substrate (113). Additionally, they work in the form of complexes with other HDACs and enzymes. These complications make it difficult to interpret the individual functions of HDACs (94). There is strong evidence of cross-talk between HDACs and other epigenetic factors in the regulation of cancer tumorigenesis (115). They can act as both tumor suppressors and cancer-causing agents depending upon their site and time of action.

HDACs have proven to be powerful enzymes that can modify a variety of proteins. The involvement of HDACs in regulating fundamental cellular functions such as proliferation, cell cycle, regeneration, apoptosis, and differentiation makes them an important target of study for disorders (114).

1.5.5.1 Class I HDACs

Class I HDACs include HDACs 1, 2, 3, and 8. This class is homologous to yeast reduced potassium dependency 3 (Rpd3) (113, 116). Class I HDACs are 350–500 amino acids in length and are expressed in all tissues, primarily localized to the nucleus (113, 117). While HDAC3 is found both in the nucleus and cytoplasm (118), some studies show that HDAC3 can be restricted to specific tissues (113). This class of HDACs is most widely studied and is plays a critical role in human biological functions. HDAC 1 and 2 have been involved in cellular functions such as proliferation and apoptosis, while HDAC 3 is involved in the DNA damage response (114, 119). Class I HDACs play a critical role in steroid hormone-dependent gene expression (120). HDACs 1 and 2 can form complexes with each other, which allows them to function both together or individually and complementary (121-123).

Many knockout (KO) studies have recognized the functional importance of individual HDACs in development (124). Growth defects have been observed in HDAC 1 KO mice, restricting

their survival to embryonic day 10.5 (125), while HDAC 2 KO mice die due to cardiovascular disorders shortly after birth (126). Class I HDACs have also been found to be elevated in several ovarian and endometrial cancers (127, 128).

1.5.5.2 Class IIa and IIb HDACs

HDACs 4, 5, 7, and 9 constitute the class IIa HDACs, while class IIb consists of HDACs 6 and 10. These classes are homologous to yeast histone deacetylases 1 and 3 (129, 130).

Class IIa HDACs can readily travel between nuclear and cytoplasmic compartments in response to post-translational modifications (131). The presence of both nuclear localization signal (NLS) and a nuclear export signal (NES) helps them to compartmentalize themselves [127] properly. Since the location of HDACs in the nucleus is considered critical for their gene silencing activities, cellular localization of this class can determine deacetylation ability (132). An extended N-terminal domain is present in addition to a C-terminal catalytic domain in the class IIa HDACs, which differentiates them from other HDAC families (133). Their N-terminal domain consists of highly conserved serine residues, which undergo signal-dependent phosphorylation, aiding in migration from the nucleus to cytoplasm (133). Due to the substitution of histidine in place of a tyrosine residue in the catalytic site of this class of HDACs, they have minimal deacetylase activity (134, 135). Instead, they act as corepressors, functioning as multiprotein complexes to influence transcription factors and other epigenetic regulators (133, 134, 136).

Class IIa HDACs are expressed in specific tissues and are associated with cell differentiation and development (119, 133). HDACs 4, 5, and 9 are expressed mainly in the heart, brain, and skeletal muscles, whereas HDAC 7 is expressed in endothelial cells in the heart, vascular system, skeletal muscle, and lungs and controls angiogenesis (137, 138). HDAC 4 KO mice display premature bone ossification, HDAC 5 or 9 KO mice show increased cardiac

hypertrophy in response to stress, and HDAC 7 KO mice exhibit a failure to form circulatory junctions during embryonic development (133). It is also seen that HDAC 5, 6, and 7 are only weakly expressed in endometrial carcinomas, suggesting they may not have a role in increasing cell proliferation (139).

In contrast to class IIa HDACs, class IIb HDACs contain two catalytic domains (132). Human HDAC 6 has 1255 amino acids and possesses an additional zinc finger motif, similar to BRAP2 (BRCA1 associated protein 2) and ubiquitin-specific proteases (USPs) (140, 141). HDAC 6 shuttles between the cytoplasm and nucleus with the help of its two leucine-rich nuclear export sequences (NES1, NES2) present at the N-terminal (141, 142). Aside from deacetylating histones, HDAC 6 also deacetylates non-histone proteins such as α-tubulin, cortactin, HSP90, β-catenin, peroxiredoxin, surviving, ubiquitin, tau, IIp45 and EGFR (141, 143). Deacetylation of α-tubulin by HDAC 6 triggers cell motility and determines microtubule stability and function (141, 144-146). HDAC 6 functions in complexes with other proteins and regulates cell migration and spreading, viral infections, misfolded protein degradation, and immune synapse formation, and are studied in several neurodegenerative diseases and cancers (142, 147, 148). The first domain of HDAC 6 shares a similarity with the N-terminal of HDAC 10. While HDAC 6 is localized primarily in the cytoplasm, HDAC 10 is localized in both the cytoplasm and nucleus (138, 149). Only one of two catalytic domains present in HDAC 10 is considered functional (150). Recent KO studies on HDAC 10 have shown its effect on chaperon-mediated autophagy (151, 152). HDAC 10 is also known to regulate DNA mismatch repair and cancer (153-155).

1.5.5.3 Class III HDACs (Sirtuins)

Class III HDACs comprise of sirtuins and are NAD+ dependent deacetylases. Their deacetylase activity is regulated by the NAD+/NADH ratio (156). They belong to the Sir2 family of

proteins and are known to be involved in cellular proliferation, migration, and angiogenesis (157). Apart from deacetylating histones, they deacetylate several non-histone proteins such as NF-κB, FOXOs, PPARϒ, PGC1-α, acetyl coenzyme-A, and α-tubulin (157). Sirtuins are linked to cellular metabolism; they sense energy levels and protect cells from metabolic stress (158). SIRT1, 6, and 7 are localized in the nucleus (159); SIRT2 is present in both cytoplasm and nucleus; SIRT3, 4, and 5 are localized in the mitochondria (158, 159). SIRTs 1 and 3 shuttle between the cytoplasm and nucleus (157). Sirtuins are involved in several critical biological functions. SIRT1 increases cell survival and life span and inhibits apoptosis (156). It has also been seen that SIRT1, 2, and 3 are involved in fat metabolism (156, 160, 161). Loss of SIRT1 is observed in obese humans and mice, leading to several metabolic disorders (159). An increase in SIRT2 levels is seen with age and may be associated with Parkinsons' Disease (162). SIRT3 also regulates oxidative stress, mitochondrial biogenesis, and aging (161). SIRT4 is a mitochondrial sirtuin that regulates cellular metabolism and the DNA damage response (163). SIRT5 is involved in various metabolic processes as well, such as glycolysis, TCA cycle, fatty acid oxidation, electron transport chain and ketone body formation (164). SIRT6 plays a critical role in sensing DNA double-strand breaks and activates processes to facilitate repair (165). SIRT7 expression is associated with cell proliferation, differentiation and the stress response. High levels of SIRT7 are associated with tumorigenesis and poor prognosis of cancers (166).

1.5.5.4 Class IV HDACs

HDAC 11 is the only member of this family and is most closely related to class I HDACs, however, the sequence similarity is too low for it to be included in the same family (167). HDAC 11 is primarily localized in the nucleus with tissue-specific expression. It is also the smallest of all the HDACs with 39kDa molecular weight (168-170). HDAC11 is also linked to immune function and tumorigenesis (170).

1.5.6 Histone Deacetylase Inhibitors (HDACi)

HDAC inhibitors, or HDACi, are compounds that have both epigenetic and non-epigenetic roles in the cell cycle (171). They inhibit HDAC activity and lead to hyperacetylation of histones. Most HDACis cause apoptosis and cell cycle arrest, playing a vital role in anti-cancer drug development. HDACis can act on specific HDACs but can also inhibit a wider array of HDACs by acting as pan-inhibitors. They can be classified into five classes depending on their chemical Zn2+-binding group to the HDACs: hydroxamic acids (hydroxamates), short-chain fatty (aliphatic) acids, benzamides, cyclic tetrapeptides, and sirtuin inhibitors (171, 172). An overview of selected HDACis and their classes are given in Table 1. (172). SAHA also known as Vorinostat, Romidepsin, Panabinostat, and Belinostat has been approved by the FDA for clinical applications (173).

Table 1. Histone Deacetylase inhibitors (172)

Class	HDAC inhibitor	Target HDACs
Hydroxamic acids	Trichostatin A	pan-inhibitors
	SAHA	pan-inhibitors
	Belinostat	pan-inhibitors
	Panabinostat	pan-inhibitors
	Givinostat	pan-inhibitors
	Resminostat	pan-inhibitors
	Abexinostat	pan-inhibitors
	Quisinostat	pan-inhibitors
	Rocilinostat	II
	Practinostat	I, II and IV
	CHR-3996	I
Short-chain fatty acids	Valproic acid	I, IIa
	Butyric acid	I, II
	Phenylbutyric acid	I, II
Benzamides	Entinostat	I
	Tacedinaline	I
	4SC202	I
	Mocetinostat	I, IV
Cyclic tetrapeptides	Romidepsin	I
Sirtuins inhibitors	Nicotinamide	III
	Sirtinol	SIRT1 and 2
	Cambinol	SIRT1 and 2
	EX-527	SIRT1 and 2

1.5.7 Histone Acetylation in the Endometrium

The menstrual cycle is the cumulative actions of various morphological and functional changes in the endometrium, which are regulated in a timely fashion by estrogen and progesterone. These changes include regeneration, proliferation, differentiation, and degradation of the endometrial tissue. These necessary cellular activities involve histone acetylation and HDAC

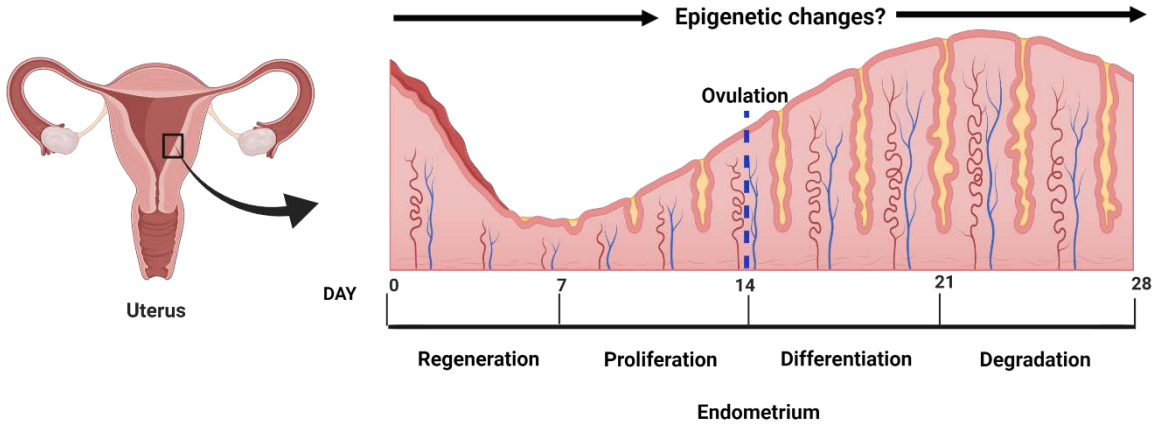

Figure 4: Epigenetic regulation throughout menstrual cycle. *Created using BioRender.com

activity. A previous study has shown changes in global histone acetylation levels throughout the menstrual cycle, suggesting the regulation of these cyclic changes involves histone acetylation and hence HAT and HDAC activity (21).

1.5.7.1 Regeneration of the Endometrium

After each menstrual cycle, the endometrial functionalis layer regenerates from the stratum basalis. It grows from an initial thickness of 0.5-1.0 mm to up to 7.0–8.0 mm in the secretory phase (28). It not only regenerates during the menstrual cycle, but also after parturition, almost complete resection, and in postmenopausal women taking hormone replacement therapy (174, 175). Endometrial re-epithelialization in humans occurs rapidly and is scar-free (34). For several years, epigenetic regulation has been linked to regeneration processes (176). HDAC inhibitors or histone acetylating agents are capable of inducing scar-free wound healing by stimulating cytokines or growth factors, which are crucial for rapid re-epithelialization (177).

The remarkable regenerative capacity of the endometrium is due to the presence of adult progenitor stem cells (178). These cells are found throughout the body and play a crucial role in the regeneration and repair of damaged tissues and the maintenance of organs. Epithelial progenitor cells and mesenchymal stem/stromal cells play an essential role in the regeneration and repair of endometrial epithelium and stroma (174). A small population of mesenchymal stem cells (MSC), a subpopulation of adult progenitor stem cells, can be found in the endometrium and are called endometrial MSCs (eMSC) (179, 180). They are highly regenerative stem cells that have similar properties to bone marrow MSCs (174). Menstrual blood also contains clonogenic, multipotent MSCs, which have a broad differentiation capacity (181).

Over the past decade, several studies have shown that epigenetics are a crucial regulator of stem cell functioning (182). Histone modification changes chromatin architecture and regulates gene expression. Since histone acetylation is an essential post-transcription modulator, it is a basic requirement in stem cell functioning and works in coordination with DNA methylation activity. The fate determination of MSCs is controlled by a complex network of transcription factors and histone-modifying enzymes (183). HDAC 1 silencing improves the efficiency of human umbilical cord MSCs in mouse models with traumatic brain injury (184); while HDAC 6 deficiency, causing acetylation of p53 K120, can induce apoptosis in MSCs (185). Global H3K9Ac level decreases, and H3K9Me2 increases during osteogenic differentiation of MSCs (186). These studies indicate the involvement of histone acetylation in the regulation of MSC function.

The endometrium has become a popular source of stem cells for reprogramming into induced pluripotent stem cells (iPSCs) (174). The MSCs derived from the endometrium and umbilical cord are being used as iPSCs in regenerative medicine, exploiting the epigenetic regulation of these cells (187, 188). HDAC inhibitors such as valproic acid (VPA), trichostatin A (TSA),

suberoylanilide hydroxamic acid (SAHA), and butyrate have been known to enhance reprogramming in iPSCs (189).

Aberrant epigenetic regulation in eMSCs has been associated with endometriosis pathogenesis (190). eMSCs are being targeted for the development of novel therapies for the treatment of endometriosis via epigenetic reprogramming (191).

Up-regulation of the global acetylation levels of H2AK5, H3K9, and H4K8 during the early proliferative phase in the adult endometrium correlates with transcription activation of several regenerative pathways (21). Although changes in histone acetylation levels and HDACs in normal to diseased conditions have been discussed and known evidence of epigenetic involvement in endometrial regeneration is available, the target genes and level of HDAC expression throughout the menstrual cycle have not yet been identified.

Histone acetylation and DNA-methylation work in a coordinated manner, decide stem cell fate and influence cancer pathogenesis (192).

1.5.7.2 Proliferation

After endometrium regeneration, estrogen from the developing follicle stimulates the proliferation of stromal and epithelial cells during the proliferative phase of the menstrual cycle (51, 174). Estrogen and progesterone surface receptors increase in number, and the rapid formation of new blood vessels, called angiogenesis, occurs to support the nutritional requirements of the growing tissue (193).

Cancer studies provide us with great insight into the epigenetic regulation of cell proliferation. HDACs can act as both suppressors and inducers of cell proliferation, depending on the gene they regulate. Inhibition of HDAC 1 reduces cell proliferation, and inhibition of HDAC 3 is associated with decreased migration of ovarian cancer cells (194).

Class I HDACs (1, 2, 3, and 8) are critical for modulating cell survival and proliferation, and HDACs 1, 2, and 3 play an important role in steroid hormone-dependent gene expression in the human endometrium (128, 195). A previous study on human cyclic endometrial tissues has demonstrated that the global acetylation levels of H2AK5, H3K9, and H4K8 are elevated in the early proliferative phase, and are associated with the transcriptional activation of proliferative and regenerative pathways (21). HDAC expression is upregulated in most endometrial cancers compared to normal cyclic endometrium (196). It is also seen that HDAC 1, 2, and 3 are strongly expressed in Ishikawa cells, whereas HDAC5, 6, and 7 are weakly expressed (139). A study on endometrial stromal sarcoma (ESS) showed that expression of HDACs 1 and 2 is higher in ESS compared to non-neoplastic stem cells, with HDAC 2 expression being slightly higher than HDAC 1. Studies also show that inhibition of HDAC 2 can lead to cell differentiation and inhibition of proliferation (197, 198). HDAC inhibitors can induce expression of p21 and p27 (endogenous cyclin-dependent kinase inhibitors), which cause cell cycle arrest and inhibit proliferation (199). Several studies have used HDAC inhibitors as anti-cancer agents to normalize cell proliferation in endometrial carcinomas (127, 128, 196, 200, 201). SAHA, m-carboxycinnamic acid Bishydroxamide (CBHA), Scriptaid, Oxamflatin, VPA, Sodium Butyrate, M344, Apicidine, Psammaplin A (PsA) and MS-275 are HDAC inhibitors that have been successfully studied in endometrial cancers and are found to induce cell cycle arrest and regulate proliferation (196).

Studies using HDAC inhibitors VPA and SAHA have also shown the association between HDACs and angiogenesis *in vitro.* The study showed that treatment with HDAC inhibitors VPA and SAHA, in combination with vascular endothelial growth factor (VEGF), increased endothelial cell sprouting (202), suggesting an association between histone acetylation and angiogenesis. Because angiogenesis is crucial for endometrial repair and re-epithelialization (203), the role of histone acetylation in angiogenesis is critical for menstrual cycle regulation.

1.5.7.3 Decidualization and Implantation

The rise in progesterone level post ovulation gives rise to morphological and functional changes in the human endometrium, a process called decidualization. These changes include the differentiation of fibroblastoid endometrial stromal cells (ESC) into decidual cells, the presence of decidual white blood cells, and vascular modifications in maternal arteries (30, 204). Decidualization initiates a cascade of events that allow the embryo to attach to the endometrium and coordinates trophoblast invasion (37). Invasion of trophoblast into the endometrial decidua requires tissue remodelling enzymes (205). Matrix metalloproteinases (MMPs) are a group of enzymes required for tissue remodelling and degrading extracellular matrix (ECM) (206, 207). During implantation and trophoblast invasion, MMP-2, and MMP-3 are expressed and secreted by the human endometrium. In contrast, the decidual stromal cells express tissue inhibitors of MMPs (TIMPs) that limit the degradation of ECM by MMPs, thus hindering trophoblast invasion. A balance between MMPs and TIMPs in the endometrium is required for the successful implantation of the blastocyst (206, 208, 209).

Decidualization, being a complicated process, requires dramatic gene expression changes in ESCs (204). These changes are associated with histone modifications, and several studies from the past two decades have shown a correlation between decidualization and histone modifications. In a previous study by *Munro et al.,* on human endometrial tissue biopsies, a statistically significant increase in H4K8 expression was seen during the mid-secretory phase, and H3K9 and H4K14/18 followed a similar trend (21). These findings correlate well with another study on ESCs cultured with estrogen and progesterone, which showed a significant increase in the acetylation of H4K8 and H3K9/14 (210). In a genome-wide analysis of histone modifications in human ESCs, H3K27ac and H3K4me3 levels were high during

decidualization. Genes with increased H3K27ac and H3K4me3 levels were found to be involved in insulin signaling pathways, crucial for the decidualization process (204).

Trichostatin A (TSA) is an inhibitor of HDAC; involved in the inhibition of HDACs Class I, II, and IV; and does not inhibit Class III HDACs (Sirtuins) (171). A comparative study between cultured ESCs and glandular cells isolated from human endometrium showed that the addition of TSA enhances the up-regulation of decidualization markers such as insulin-like growth factor binding protein-1 (IGFBP-1) and prolactin, as regulated by 17β-estradiol (E2) plus progesterone (P4) (210). This study showed the involvement of histone acetylation and the connection of HDACs to the decidualization of the endometrium, suggesting that TSA acts as an enhancer of the decidualization process. In contrast, a decade later, another study showed that treatment of human ESCs with TSA had an inhibitory effect on trophoblast invasion. The ESCs treated with TSA showed increased TIMP-1 and TIMP-3 expression, while the expression of MMPs was decreased. This study associated histone acetylation with the disruption of trophoblast invasion and suggested that HDACs are critical for implantation (206). A recently published study on mice demonstrated that loss of HDAC 3 in the uterus of mice leads to implantation failures. Interestingly, HDAC 3 is usually the least frequently expressed HDAC in the endometrial tissue (128, 211). In the study, HDAC 3 was shown to be involved in transcription activation of *COL1A1* and *COL1A2* (collagenase) genes in humans and *col1a1* and *col1a2* genes in mice (211). Collagenases are involved in endometrial remodelling and trophoblast invasion during implantation (206). The cumulative findings of these studies suggest that HDAC 3 could be crucial for the implantation process, but in a limited capacity. A tightly controlled relationship between HATs and HDACs is essential for proper decidualization and successful implantation.

1.5.8 HDACs and Endometrial Pathologies

The highly timed molecular events discussed above, if not appropriately regulated, lead to a spectrum of endometrial pathologies such as endometriosis, endometrial cancers, abnormal uterine bleeding, and infertility.

Studies have found the involvement of histone acetylation, in conjunction with other epigenetic modulators, in several human malignancies (114, 154, 155, 212). High expression of class I HDACs in some tumors has been linked to unfavorable prognosis, with the exception of breast cancer in which it has a favorable prognosis. On the other hand, the upregulation of class II HDACs is associated with a favorable prognosis in human tumors (213). One of the earliest studies on endometrial stromal sarcoma (ESS) showed that HDAC 2 expression is upregulated in ESS compared to the non-neoplastic endometrial stroma (197). They also showed that HDAC 1 expression is generally lower than HDAC 2, and inhibition of HDAC 2 using valproate affects cell differentiation by increasing the expression of cell cycle regulators (197). Later, a study on endometrial adenocarcinomas showed a decrease or complete loss of epithelial HDAC 1 protein expression compared to normal endometrium (128). Alternatively, a study on endometrial carcinomas (EC) showed that HDAC 1, 2 and 3 expression levels are increased in ECs compared to healthy endometrial tissues (127). Although this study did not account for the cycle stages of the endometrium in analysis, it still showed similarity with earlier studies, in that HDAC 2 is the most expressed in the endometrium, while HDAC 3 is the least expressed (127, 128). An immunohistochemical study on endometrial carcinomas showed that HDAC 1, HDAC 2, and Ki-67 (a cellular marker of proliferation) are higher in endometrial carcinomas than in normal endometrium. The treatment of endometrial carcinoma cell lines with HDAC inhibitors TSA and apicidin reduces cell proliferation and increases p21 expression. Apicidin also reduces cyclin D1 and CDK4 expression (198, 214). HDAC

inhibitors Vorinostat, Romidepsin (FK228), and LBH589 help induce cell cycle arrest in endometrial carcinomas (214-216). Progesterone induces differentiation in endometrial cells and inhibits proliferation through the action of progesterone receptors (PR). Therefore, the loss of PR also leads to endometrial carcinomas. Treatment of ECC-1 and Ishikawa H cells with the HDAC inhibitor LBH589 has been shown to increase the number of PRs, which in turn regulates cell differentiation and induces cell cycle arrest (217, 218). While most HDACs are upregulated in ECs, recent studies have shown that SIRT 6 induces apoptosis and inhibits proliferation in endometrial cancer cells by repressing survivin (219). Expression levels of SIRT 1, SIRT 2, SIRT 4, and SIRT 5 are seen to be downregulated, while SIRT 7 is significantly upregulated in ECs compared to non-neoplastic cells. Interestingly, this study showed no significant difference in SIRT 6 and SIRT 3 levels between ECs and non-neoplastic cells (220).

Histone acetylation and aberrant levels of HDACs are also associated with endometriosis. HDAC 1 expression is seen to be significantly elevated during endometriosis compared to normal endometrium, and the level also correlates with low acetylation levels of H3 and H4 (195, 221). It was seen again in a later study that HDAC 1 and 2 expression levels were high in endometriotic stromal cells compared to healthy endometrial stromal cells. Differential expression of HDAC 1 and 2 was observed based on lesion type and localization in endometrioid cells (222). A comparative study between ectopic and eutopic endometrial samples taken from women who have endometriosis showed that HDAC 1 gene expression was high in ectopic tissues while HDAC 2 expression levels were high in eutopic tissue samples. Expression of HATs P300 and CREBBP did not change significantly in endometriosis tissue samples compared to standard samples, while PCAF expression was high in the ectopic tissue (223). It should be noted that this study used total tissue samples with minimal sample size, and the total sample contained varied cell populations from various endometrial cycle

stages. The anti-inflammatory effects of SIRT1 have also been investigated in endometriosis. This study compared the action of SIRT1 and its activator resveratrol in endometriotic stromal cells and healthy endometrial stromal cells. They found that the activation of SIRT1 suppresses inflammatory responses in endometriotic stromal cells, while inhibition of SIRT1 can trigger inflammatory responses, suggesting its crucial role in maintaining healthy endometrial stromal cells (224).

HDACs also play a crucial role in implantation and decidualization. Loss of HDAC 3 is linked to decidualization defects and implantation failure in mice (225). HDAC inhibitor TSA in endometrial stromal cells promotes extracellular matrix invasion and helps with embryo implantation (206).

Since most studies on endometriosis and other endometrial pathologies compare healthy and diseased tissue samples, it is hard to tell if the epigenetic aberrations are the cause or effect of various endometrial pathologies.

1.5.9 Conclusion

Histone acetylation is a fundamental regulator of chromatin structure and gene expression. Many studies on endometrial tissues and cell lines have shown the involvement of aberrant levels of histone acetylation and HDAC activity in endometrial pathologies, especially endometrial carcinomas and endometriosis. The majority of HDACs studied seem to be elevated in endometrial carcinomas compared to the normal endometrium (127, 222). High levels of HDACs are also associated with endometriosis in many women. Evidence suggests that histone acetylation and HDACs are involved in various endometrial pathologies (195, 200, 222, 225, 226). However, most of these studies have not given any regard to the cyclic nature of the endometrium. HDACs are being studied as potential therapeutic targets for endometrial carcinomas and endometriosis; their effect reversed using HDAC inhibitors (200, 226). Global

histone acetylation levels of H2AK5, H3K9, and H4K8 are found to be elevated in the early proliferative phase of the endometrium, then decline until ovulation. The acetylation levels in H4K8 rise significantly post ovulation, and a similar trend is observed in H3K9 as well as H4K14 and 18. This study suggests that histone acetylation trends seem to follow a cyclic pattern in coordination with menstrual cycle events that require transcriptional activation and silencing (21). HDACs function in conjunction with other epigenetic modulators, regulating molecular modifications in the endometrium (223). Studying the general levels of HDACs and histone acetylation in normal cyclic endometrium will give us insight into individual functions and targets of individual HDACs. Because histone acetylation is a reversible epigenetic mark, studying its regulation will eventually help us to develop targeted therapies to improve the menstrual health of women.

1.6 Aim

Hypothesis: Epigenetic modifications play a vital role in regulating the endometrial cycle. Histone acetylation, which is generally associated with gene activation, is a common epigenetic mark and is regulated by HATs and HDACs, which include SIRTs. Temporal global histone acetylation levels have been observed throughout the menstrual cycle in a previous study in our lab (227). HDAC inhibitors have also been studied in various endometrial pathologies such as endometrial cancer and endometriosis.

We hypothesis that histone modifications are essential regulators of cyclic changes in the endometrium and that steroid hormones temporally regulate HDACs.

Aims

1. To examine the mRNA profile of histone deacetylases (HDACs) in human endometrium during the menstrual cycle

2. To determine the localization and protein profile HDACs in human endometrium during the menstrual cycle

3. To investigate changes in the mRNA and protein expression of HDACs in response to ovarian steroids, in *in vitro* endometrial epithelial and stromal cells.

2 Materials and Methods

2.1 Charcoal stripping Fetal Bovine Serum (FBS)

Gibco FBS (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was charcoal stripped to prepare for hormone treatment. 2.5gm of activated charcoal (Sigma -Aldrich, USA), 0.25gm of Dextran from Leuconostoc mesenteroides (Sigma -Aldrich, USA), and 2.5ml 1M TRIS pH7.4 was mixed with MilliQ water to make up 250mL. The solution was stirred for at least 30 minutes at 4°C before use. The solution was divided and centrifuged in 50mL falcon tubes at 3500 rpm at room temperature for 10 minutes. The supernatant was aspirated and 500mL FBS was added (divided equally in the falcon tubes). The solution was incubated in a pre-heated water bath at 55°C for 30 minutes and periodically swirled. The solution was centrifuged at 3500 rpm at room temperature for 10 minutes. FBS was pipetted and filter sterilized using Corning(R) bottle-top vacuum filters, polyethersulfone membrane, pore size 0.22 μm, membrane size 63x63 mm, funnel capacity 500mL (Sigma-Aldrich, USA). The charcoal-stripped FBS was divided into 50 mL aliquots and stored at -20°C.

2.2 Cell culture

Three cell lines were used for the experiments: Human Endometrial Stromal Cells (HESC)(228) and Acanthosis Nigricans 3rd attempt (AN3) and RL95-2 were purchased from ATCC® (Old Town Manassas, Virginia, United States). Details about the cell lines are mentioned in Table 2. The cells were grown in Gibco Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1:1) phenol-free (Thermo Fisher Scientific, Waltham, Massachusetts, United States). It was supplemented with 10% Charcoal stripped FBS and 1% antibiotic Penstrep, including penicillin and streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The frozen cells were thawed and grown in T175 (Falcon™

Tissue Culture Treated Flasks) (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with 18 mL medium. The cells were incubated in humidified cell culture incubators at 37°C and 5% CO_2. The media was changed the next day to clear the Dimethyl Sulfoxide (DMSO) present in the freezing medium. Once confluent to 90-100%, the cells were split four ways in T175 flasks. The media was changed on alternate days, and the cells were washed with sterile 1XPBS every time. All cell culture work was performed in cell culture hoods following aseptic culture techniques.

Table 2. Cell Lines

Cell Line		
HESC	Cell Type:	Fibroblast immortalized with hTERT
	Origin:	The cell line was obtained from an adult female with myomas and was immortalized by infection with supernatant from the packaging cell line pA317-hTERT (Geron Corp.; Menlo Park, CA)
	Subculture:	Cells with confluency 80-90% were split at 1:4 ratio using 1X 0.25% Trypsin (Thermo Fisher Scientific, Waltham, Massachusetts, United States) into T175 flasks.
	References:	T HESCs ATCC® CRL4003™
AN3	Cell Type:	Human endometrial adenocarcinoma
	Origin:	The cell line was derived from a metastatic lesion in the lymph node of a patient with endometrial carcinoma by C. J Dawe and associates (229).
	Subculture:	Cells with confluency 80-90% were split at 1:4 ratio using 1X 0.25% Trypsin (Thermo Fisher Scientific, Waltham, Massachusetts, United States) into T175 flasks.
	References:	ATCC® HTB-111™ , (229)
RL95-2	Cell Type:	Human endometrial carcinoma
	Origin:	Derived from Grade 2 moderately differentiated adenosquamous carcinoma of the endometrium by D. L. Way and associates (230).
	Subculture:	Cells with confluency 80-90% were split at 1:4 ratio using 1X 0.25% Trypsin (Thermo Fisher Scientific, Waltham, Massachusetts, United States) into T175 flasks.
	References:	(ATCC® CRL-1671™) (230)

2.3 Hormone Treatment of Cells

250000 cells were plated in each well of 6 well plates. Once cells were 70% confluent, they were treated with β- estradiol (E2) final concentration of 0.01µM in each well, and progesterone (P4) final concentration of 1µM in each well. The cells were treated in triplicates first with E2 for 24 hours and then with E2+P4 for 24 hours, 48 hours, and 72 hours. For each triplicate, treatment cells were also exposed to 1µM Trichostatin A (TSA) (Sigma-Aldrich, USA). Untreated controls were included for each treatment interval. Hormone stocks were prepared in analytical grade ethanol using hormone powders (Sigma -Aldrich, USA). TSA was prepared in DMSO as per manufacturers' instructions. For control cells, ethanol and DMSO alone were used the same amounts as in hormone stocks. The stocks were diluted in PBS to prepare working solutions before diluting in media to the final required concentration. 2mL of media was added to each well. Each treatment was performed three independent times. The plate layout for the hormone treatment is given in the figure below.

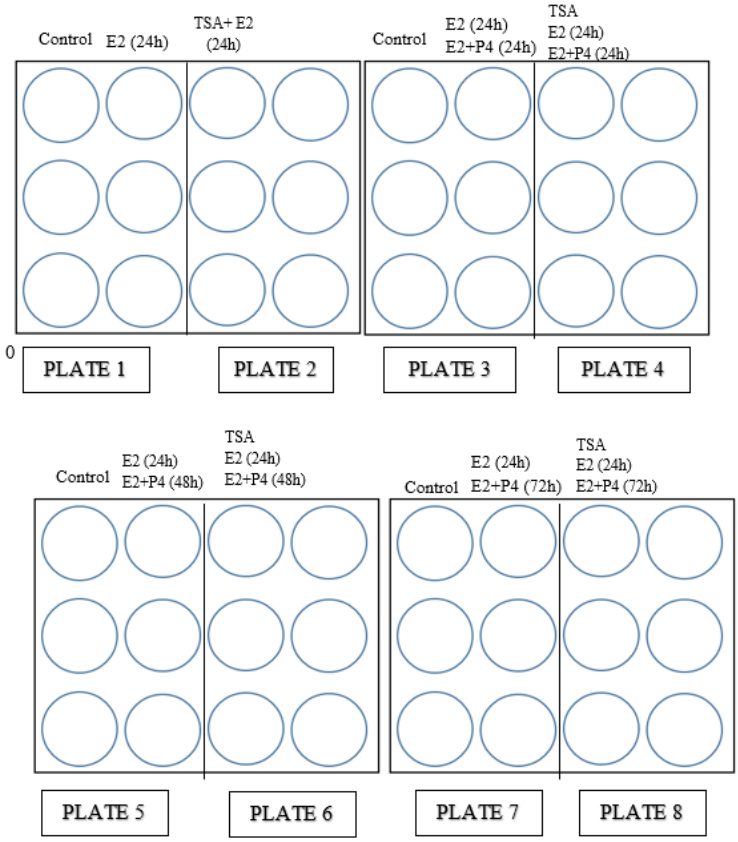

Figure 5: Plate Layout for hormone treatment

2.4 Cell harvesting

Cells were collected according to the timed intervals of E2 (24 hours), E2+P4 (24 hours), E2+P4 (48 Hours) and E2+P4 (72 hours). 1mL media was collected for further experiments and the rest was discarded. The cells were washed with ice-cold 1XPBS and treated with Trizol® LS reagent (Life Technologies, Carlsbad, California, United States) or RIPA lysis buffer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) as required. The cells were scraped off using plastic cell scrapers and collected in Eppendorf tubes. Separate scapers and tips were used for each well.

2.5 Tissue Samples

Endometrial tissue biopsies collected at different stages of the menstrual cycle from premenopausal healthy women were used for the experiment. Women with any known endometrial pathology or undergoing hormonal contraception within three months of sample collection were excluded from the study. The samples with any histopathological evidence of abnormality were excluded as well. These tissue samples were provided by Dr. Anna Ponnampalam at the Liggins Institute. The stages were pre-determined using histological techniques.

2.6 RNA Extraction

RNA was extracted from frozen endometrial tissue samples using Trizol® LS reagent (Life Technologies, Carlsbad, California, United States) according to manufacturers' instructions. The frozen tissue samples were crushed using a mortar and pestle and powdered tissue was added to Eppendorf tubes containing 1ml trizol. Tissue was kept frozen using liquid nitrogen to avoid degradation. A ball bearing was added and samples were lysed using a tissue lyser at

30 frequency for 30 seconds using pre-cooled tissuelyser adapters. The mixture was then transferred to separate tubes.

RNA extraction from cell cultures was also done using Trizol® LS reagent (Life Technologies, Carlsbad, California, United States). The media was pipetted and stored for further experiments, cells were washed once with ice-cold PBS (1mL), and 1mL trizol was added to each well in 6-well culture plates. Plates were scraped briefly and trizol was pipetted up and down to homogenize cells which were then collected in Eppendorf tubes. Both the tissue and cell lysates were treated in the same manner from this point onwards

The samples were left at room temperature (15-30°C) for 5 minutes to allow complete dissociation. 0.2mL of chloroform (Sigma-Aldrich, USA) was added per 1mL of trizol, tubes were vortexed vigorously for 15 secs and then incubated at room temperature for 2-3 minutes. The samples were centrifuged at 12000g for 15 mins at 4°C. The aqueous phase (top layer) was transferred to a fresh Eppendorf tube without disturbing the organic phase. Isopropanol was added (50% of the total solution) to the tubes and they were incubated at 4°C for 20 mins. Samples were centrifuged again at 12000g for 15 mins at 4°C. The supernatant was discarded, and pellets were washed twice using 1 mL of 75% ethanol per mL of trizol and centrifuged at 10000 g for 5 minutes at 4°C in each wash. The pellets were spun at maximum speed for 30 seconds at 4°C to remove ethanol. The pellets were then air-dried at room temperature until all remaining ethanol had evaporated. The RNA pellets were resuspended in approximately 10µL of nuclease-free water, depending on pellet size. The concentration of samples was determined using the NanoPhotometer® N60 (Implen, Munich, Germany), and RNA integrity was assessed using Agilent 2100 bioanalyzer (Agilent Technologies, New Zealand).

2.7 RNA Integrity

The RNA integrity was determined using Agilent 2100 bioanalyzer (Agilent Technologies, New Zealand) according to manufacturer protocol. The electrodes were cleaned using RNaseZap™ (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and Nuclease free water. The dye, pre-prepared aliquotes of gel, and marker brought to room temperature. The dye was spun down and 1µL was added to the gel. The mixture was vortexed and then centrifuged at 13000g at room temperature for 10 minutes. The gel-dye mix was stored in the dark at room temperature until needed. 1.2µL aliquots of 1:10 diluted RNA were taken in 1.5mL tubes and heat-denatured at 70°C for 2 minutes. Samples were centrifuged briefly before use. RNA nanochip was set up on the chip priming station and the syringe plunger was pulled up till 1mL. 9µL of the gel-dye mix was added to the well marked **G** (third row, fourth column) and the chip priming station was closed. The plunger was pressed until held by the clip. After 30 seconds, the clip was released and the plunger was slowly pulled up to 1mL. 9µL of the gel-dye mix was added to each of the other two wells marked G. 5µL of RNA 6000 Nano marker (green) (Agilent Technologies, New Zealand) was added to all remaining wells. One 1µL ladder was added to the ladder well and 1 µL samples were added to each of the remaining wells. The chip was vortexed at 2400 rpm for 1 minute in Ika MS3 vortexer (Sigma-Aldrich, USA). The chip was then placed in the Agilent 2100 bioanalyzer and analyzed using an assay for eukaryote RNA. RNA integrity number (RIN) seven and above were considered a good quality for experiments.

2.8 Reverse Transcription

High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used to perform reverse transcription of RNA into cDNA. 2µg of RNA was used as a starting material. 1µL each of DNase 1 and

10X DNase 1 buffer from the DNase I Amplification Grade kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) were added to each sample tube and the quantity was made up to 9µL using nuclease-free water (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The samples were incubated at room temperature for 15 minutes to allow for DNase activity. 1µL of EDTA was added to each sample and tubes were incubated at 65°C for 10 minutes to inactivate the DNase. 2µL of 10X reverse transcriptase buffer, 0.8µL of 25X dNTP Mix, 2µL of 10X random primers, and 1µL of Multiscribe Reverse transcriptase were added to each tube. The volume was made up to 20µLwith nuclease free water. One extra sample tube was used in which all the reagents were added except reverse transcriptase as a negative control. The reaction was run in BioRad DNA engine Perrier thermal cycler (BioRad Laboratories, Hercules, California, United States) at 25° C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and then held at 4°C until stored. The cDNA was stored at -20°C until used for a quantitative reverse transcription-polymerase chain reaction.

2.9 Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was conducted on cDNA samples to quantify RNA expression levels. QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific, Waltham, Massachusetts, United States), MicroAmp™ Optical 384-Well Reaction plate (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and SYBR™ Select Master Mix (Applied Biosystems™, Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used according to manufacturer protocol. 2µL of each cDNA sample was added to one tube and diluted to 1:10, 1:100, and 1:1000 for use as standards for the experiment. The primer pairs were taken from the primer bank (details described in the table below). For each reaction, 0.5µL of each forward and reverse primer was used with 5µL of SYBR™ Select

Master Mix, 1µL of nuclease-free water and 3µL of 1:20 diluted cDNA sample. The samples were pipetted in the PCR plate using epMotion® 5070 (Eppendorf, Hamburg, Germany), with the settings for the master mix liquid type was changed to glycerol. The reaction was run under the following settings: QuantStudio™ 6 Flex System, 384-well, relative standard curve, SYBR, standard. The reaction was set up with the activation stage at 95°C for 2 minutes, followed by 45 cycles of amplification, denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 30 seconds. All samples were analyzed in triplicates with standards for each gene. Primers for HDACs and SIRTs designed by Dr. Sheryl Kirsty Munro were used from her work on HDACs (227), except for HDAC 2 primers which were obtained from the primer bank (231) (ID#293336690c1). Primers for housekeeping genes were already available with the lab. Primer pairs used are summarized in the table below.

Table 3. Primer Pairs

Primers	Forward 5´-3´	Reverse 3´-5´	Amplicon size (bp)	Source
HDAC1	GGAAATCTATCGCCCTCACA	TTGCCACAGAACCACCAGTA	206	(227)
HDAC2	ATGGCGTACAGTCAAGGAGG	TGCGGATTCTATGAGGCTTCA	112	(231)
HDAC3	TGGCTTCTGCTATGTCAACG	TTGATAACCGGCTGGAAAAG	297	(227)
HDAC4	CCTCTACACATCGCCATCCT	GGCTGCTCCAGTAAGACCAT	237	(227)
HDAC5	GTAGCCATCACCGCAAAACT	GTCCTCCACCAACCTCTTCA	199	(227)
HDAC6	TATCTGCCCCAGTACCTTCG	GGACATCCCAATCCACAATC	242	(227)
HDAC7	GGCTGCTTTCAGGATAGTCG	TTCATCAGTTGCTGCGTCAT	162	(227)
HDAC8	TTATGACTGCCCAGCCACTG	TTCCCAGGACAGCATCATTG	188	(227)
HDAC9	GACTCACTCTTCCCCTGCTG	CCAGTTTCTTGCAGTCGTGA	192	(227)
HDAC10	CTTCTCCTGGCACCGCTATG	CCGATGGCTGAGTCAAATCC	234	(227)
HDAC11	ATCACGCTCGCCATCAAGTT	TCATCCTCTGTGCCCCACTC	224	(227)
SIRT1	GCTGATGAACCGCTTGCTAT	ATGGGGTATGGAACTTGGAA	174	(227)
SIRT2	GACTTTCGCTCTCCATCCAC	GAGTAGCCCCTTGTCCTTCA	201	(227)
SIRT3	AGCAACCTCCAGCAGTACGA	CGACACTCTCTCAAGCCCATC	216	(227)
SIRT4	ACCTGGGAGAAACTCGGAAA	TCAGGACTTGGAAACGCTCT	178	(227)
SIRT5	ATGCGACCTCTCCAGATTGT	GTCGGAACACCACTTTCTGC	206	(227)
SIRT6	GCAGTCTTCCAGTGTGGTGTTC	CGCTCTCAAAGGTGGTGTCG	140	(227)
SIRT7	CCATTTTCTCACTGGCGACT	CCTTTTTGTGCGTTTTGTGC	173	(227)
YWHAZ	CCGTTACTTGGCTGAGGTTG	CAGGCTTTCTCTGGGGAGTT	189	(227)
RPLO	AGAAACTGCTGCCTCATATCCG	CCCCTGGAGATTTTAGTGGTGA	223	(227)
RPL13A	GCCCTACGACAAGAAAAAGCG	TACTTCCAGCCAACCTCGTGA	117	(227)

2.10 Standards and Standard Curves

2µL of each endometrial cDNA samples were combined to create standards of 1:10, 1:100, and 1:1000 dilutions. The standards were tested using qRT-PCR against housekeeping genes YWHAZ, RPLO, and RPL13A, as well as for each of the other genes. Standard curves were generated, and R^2 values were determined using excel. R^2 values above 0.99 were considered suitable for the experiment.

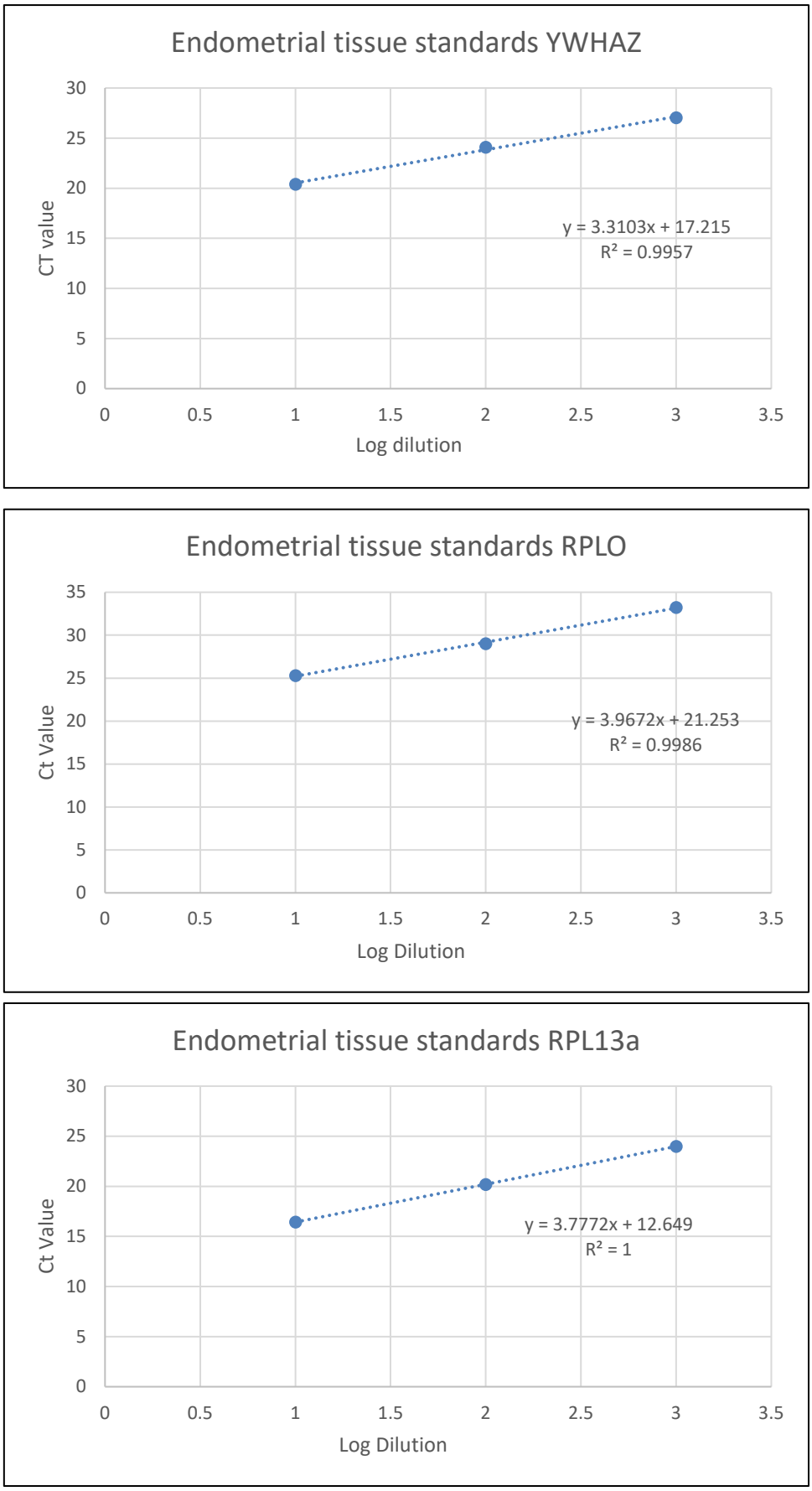

Figure 6: Standard curves for housekeeping genes in endometrial tissue samples

2.11 Statistical Analysis of qRT-PCR

The ΔΔCT method was used for statistical analysis of gene expression. Average CT values of the sample triplicates of cDNA samples from tissue biopsies were normalized against the geometric mean of the three housekeeping genes (YWHAZ, RPLO, and RPL13a), while for cell samples two housekeeping genes (YWHAZ and RPL13A) were used. The samples were divided according to the tissue uterine cycle stage and the treatment type Estradiol 24hrs (E24), Estradiol+ Progesterone 24hrs (EP24), Estradiol+ Progesterone 48hrs (EP48) and Estradiol+ Progesterone 72hrs (EP72), respectively. ΔCT value was calculated using the following formula:

$$\Delta CT = CT\ target - Geomean$$

CT target is the mean CT value of the sample under investigation. The geomean of the housekeeping genes was subtracted from this to determine ΔCT. One of the samples was taken as reference, and ΔΔCT was calculated using the following formula:

$$\Delta\Delta CT = \Delta CT\ target - \Delta CT\ reference\ sample$$

The range for the target gene expression relative to the reference sample was calculated as $2^{-\Delta\Delta CT}$.

The $2^{-\Delta\Delta CT}$ values of samples were plotted against the endometrial cycle stage. Graphpad Prism version 8.2.1. (San Diego, CA, USA) was used for statistical analysis. To determine the temporal expression of HDACs during the menstrual cycle in endometrial, One-way non-parametric ANOVA, followed by unpaired t-tests was done to determine statistical significance. To determine the effects of steroid hormones on HDAC expression in cells *in vitro*, two-way ANOVA was performed, and further significance was determined within each treatment group, and intervals using multiple comparisons uncorrected Fisher's LSD.

2.12 Protein Extraction

The protein extraction from frozen tissue samples was done using RIPA buffer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) according to manufacturer protocol. Tissue samples were powdered using a sterile mortar and pestle, added to Eppendorf tubes, and weighed. The tissue was kept frozen using liquid nitrogen. 20µL of Halt™ Protease Inhibitor Cocktail (EDTA-Free (100X)) (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was added per 1mL of RIPA lysis buffer. Approximately 20µL of RIPA lysis buffer and Halt™ solution were added per microgram of tissue. A ball bearing was added to the samples which were lysed using a tissue lyser at 30 frequency for 10 minutes using pre-cooled tissue lyser adapters.

RIPA-Lysis buffer was also used for extracting protein to use in cell cultures. The media was pipetted and stored for further experiments, and cells were washed once with ice-cold PBS (1mL). 1mL of RIPA and Halt™ mix was added to each well. Plates were scraped briefly, and cell lysate was pipetted up and down to homogenize cells, which were then collected in Eppendorf tubes. Both the tissue and cell lysates were treated in the same manner from this point onwards.

The samples were incubated at 4°C on an orbital shaker for 2 hours and then centrifuged for 20 minutes at 12000 rpm at 4°C. The supernatant was collected in fresh tubes, and the concentration of samples was determined using Direct Detect® Infrared Spectrometer (MilliporeSigma, Burlington, Massachusetts, United States). The lysis buffer was used as a blank. The measurement was done using analysis method 2.

2.13 SDS-PAGE

Nu-Page 4-12% Bis-tris 15 lane mini gels were used to perform SDS-Page according to manufacturer protocol. 10ug of each sample was loaded in each lane with 5uL of NuPAGE™ LDS Sample Buffer (4X), 2µL of NuPAGE™ Reducing Agent (10X) (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and enough autoclaved MiliQ water to make up the total volume of 20µL. The sample mix was denatured for 10 minutes at 70°C. NuPAGE MOPS SDS Running Buffer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used to run the samples on PowerPac™ (BioRad Laboratories, Hercules, California, United States) at 200V for 1 hour. 5µL of MagicMark™ XP Western Protein Standard (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used to determine the protein bands. NuPAGE™ Transfer Buffer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used to transfer the proteins to Immobilon-FL PVDF Membrane (MilliporeSigma, Burlington, Massachusetts, United States) using PowerPac™ (BioRad Laboratories, Hercules, California, United States) at 50V for 1 hour. Membranes were then stained with Revert™ 700 Total Protein Stain (LI-COR Biosciences, Lincoln, Nebraska, United States) for 5 minutes using the single color western blot protocol. The membranes were rinsed twice with Revert™ 700 Wash Solution (LI-COR Biosciences, Lincoln, Nebraska, United States) and then with MiliQ water to remove the excess staining. The membranes were dried and then visualized in the 700 nm channel with an Odyssey® imaging system, Classic Fc (LI-COR Biosciences, Lincoln, Nebraska, United States).

2.14 Western Blot

The HDAC protein expression was detected using western blot. Following total protein imaging, the membranes were placed in 100% methanol and then rinsed with MiliQ water until the methanol was washed off. The non-specific binding was blocked using Intercept Tris-

buffered saline (TBS) Blocking Buffer (LI-COR Biosciences, Lincoln, Nebraska, United States) for 1 hour at room temperature. Following blocking, the membranes were incubated in primary antibody dilutions prepared in the blocking buffer on a rocking platform overnight at 4°C. The membranes were then washed four times with 1X TBST for 5 minutes, and incubated in secondary antibody diluted in blocking buffer for 1 hour at room temperature on a rocking platform, protected from light using aluminium foil. The membranes were then washed again with 1X TBST four times for 5 minutes and rinsed with 1X TBS before imaging. Membranes were protected from light at all times following secondary antibody incubation. Antibodies were optimized using two endometrial protein samples and a placental villous protein sample according to the dilutions used previously by Dr. Sheryl Munro and manufacturers' recommendations. All the antibodies and dilutions used are summarized in the tables below.

Table 4. Primary Antibodies

Target	Antibody	Supplier	Species	Band Size	Concentration
HDAC1	Ab19845	Abcam	Rabbit	55 kDa	1:2000
HDAC2	Ab51832	Abcam	Mouse	55 kDa	1:1000
HDAC3	Ab16047	Abcam	Rabbit	50 kDa	1:3000
HDAC4	Ab32534	Abcam	Rabbit	119 kDa	1:5000
HDAC5	Ab55403	Abcam	Rabbit	122 kDa	1:500
HDAC7	Ab53101	Abcam	Rabbit	103 kDa	1:500
HDAC8	Ab187139	Abcam	Rabbit	42 kDa	1:500
HDAC9	Ab109446	Abcam	Rabbit	111 kDa	1:15000

Table 5. Secondary Antibodies

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

2.15 Protein Expression Analysis

Normalization was performed separately for each blot using Revert™ 700 Total Protein Stain. The target protein bands were normalized against the total amount of sample protein per lane. The target and total protein in each lane were quantified, and the normalization factor was calculated for each sample using total protein signal (TPS) with the formula below.

$$Lane\ Normalization\ Factor = \frac{TPS\ for\ Each\ Lane}{TPS\ from\ the\ lane\ with\ the\ highest\ TPS}$$

The normalized signal was calculated for each sample to be used for relative quantitative comparison.

$$Normalization\ Signal = \frac{Target\ band\ signal}{Lane\ Normalization\ Factor}$$

The normalized 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.

3 Results

3.1 HDAC Gene expression changes during menstrual cycle

The relative gene expression was studied across four cycle stages: proliferative (P) (n=10), early secretory (ES) (n=3), mid secretory (MS) (n=2) and late secretory (LS) (n=6). The mRNA expression levels of HDACs were normalized against three housekeeping genes YWHAZ, RPLO, and RPL13A and plotted as Mean±SEM against the menstrual cycle stages. The statistical significance was determined using one-way ANOVA followed by unpaired t-tests on GraphPad Prism 8.2.1. Most of the HDACs were significantly upregulated during the early secretory phase.

In class 1 HDACs, HDAC1 and HDAC8 were significantly upregulated during the early secretory phase compared to proliferative (*P<0.05) and late secretory (**P<0.01) phases (Figure 7 a and c), while HDAC2 was significantly upregulated during mid secretory phase. HDAC3 had little or no expression in the endometrial tissues across the cycle.

Class 2a HDACs 5 and 7 were significantly upregulated during the early secretory phase (Figure 8 b and c), while HDAC9 was upregulated during the mid-secretory phase (Figure 9 d), compared to other cycle stages. No significant differences were observed in HDAC4 mRNA expression during the cycle.

In class 2b a significant upregulation in HDAC10 expression level was observed in the mid secretory phase compared to proliferative and early secretory stages, while there was a trend towards upregulation against the above phases in the late secretory stage (Figure 9 b).

In class 3 HDACs SIRT2, 3, 4 and 7 showed significant changes within the menstrual cycle. SIRT 2 was significantly upregulated in early (*P<0.05) and mid secretory phase (**<0.01)

(Figure 10 b). SIRT3 and 4 were significantly upregulated in the early secretory phase (*P<0.05) (Figure 10 c-d). SIRT5 (P=0.054) and SIRT6 (P=0.084) followed the similar treat with near significant increase in expression during the early and mid secretory phases respectively (Figure10 e-f). SIRT7 was significantly upregulated as well during the early secretory phase (*P<0.05)

Class 4 HDAC 11 was significantly upregulated as well during the early secretory phase (*P<0.05).

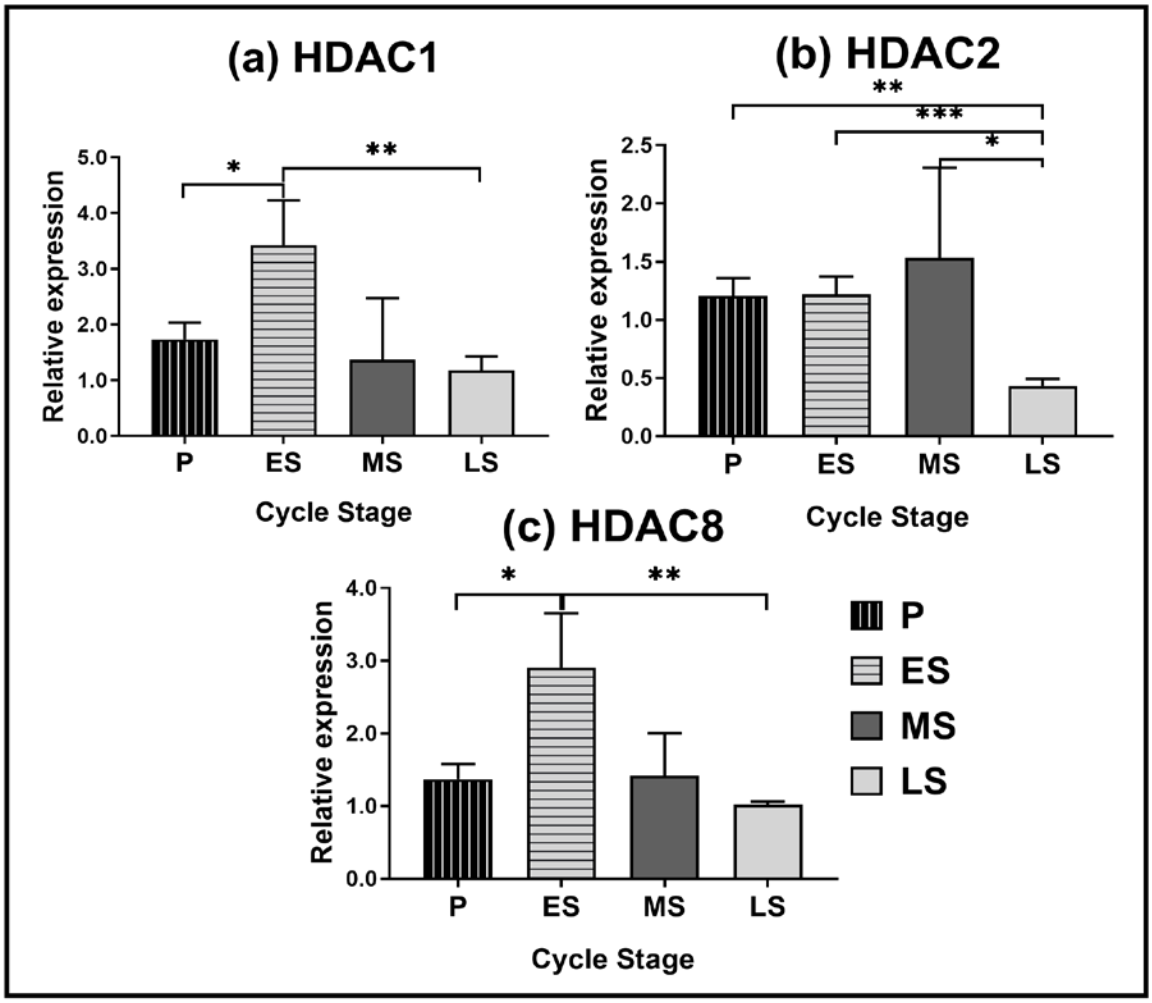

Figure 7: Relative mRNA expression of class 1 HDACs during menstrual cycle

Real time qPCR results for class 1 HDACs in human endometrium during the menstrual cycle. 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

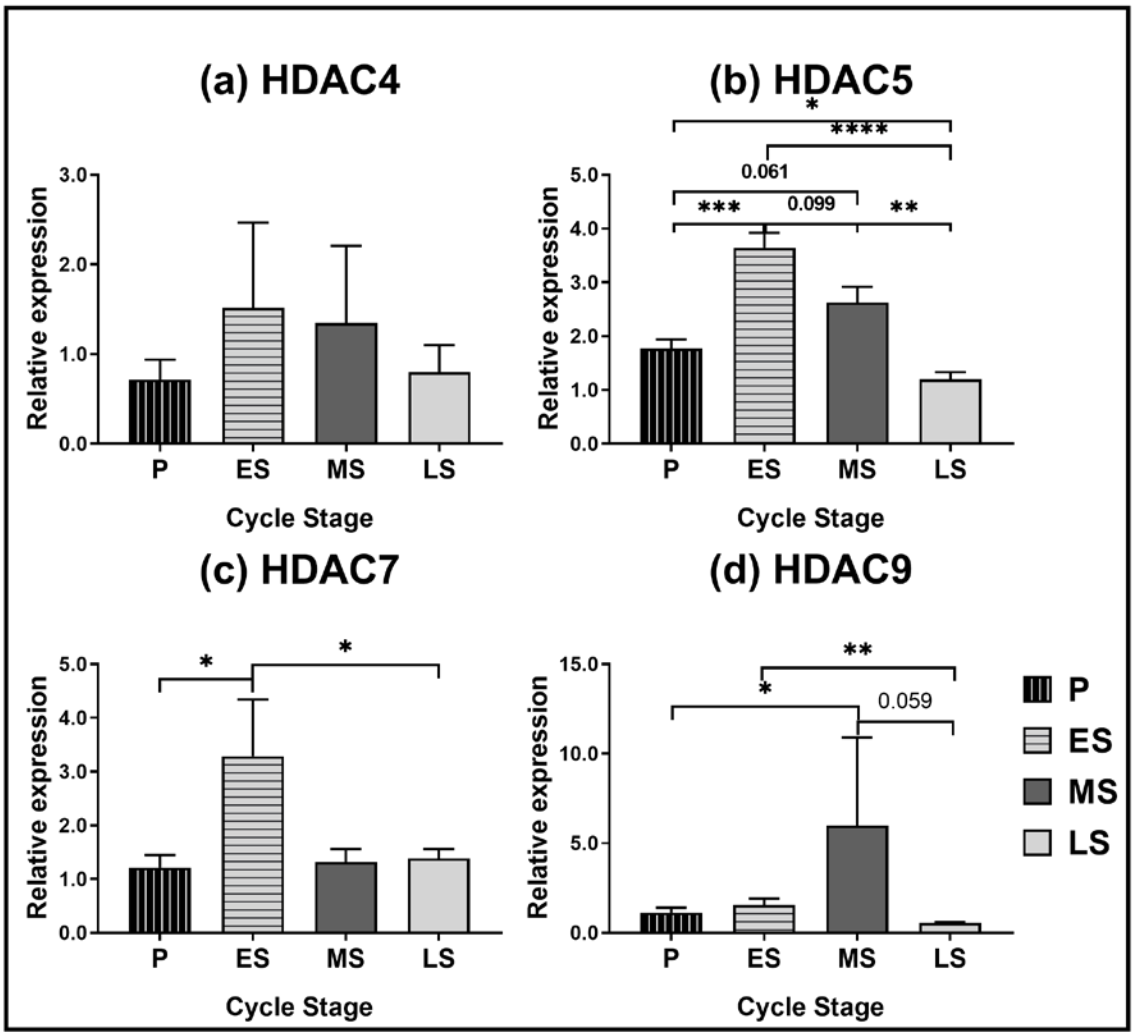

Figure 8: Relative mRNA expression of class 2a HDACs during menstrual cycle

Real time qPCR results for class 2a HDACs in human endometrium during the menstrual cycle. 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

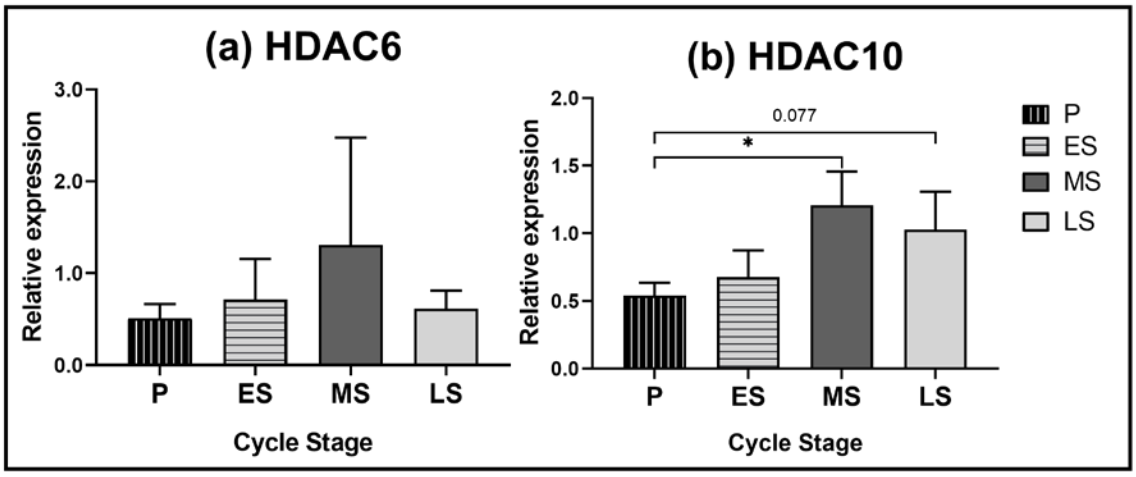

Figure 9: Relative mRNA expression of class 2b HDACs during menstrual cycle

Real time qPCR results for class 2b HDACs in human endometrium during the menstrual cycle. 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

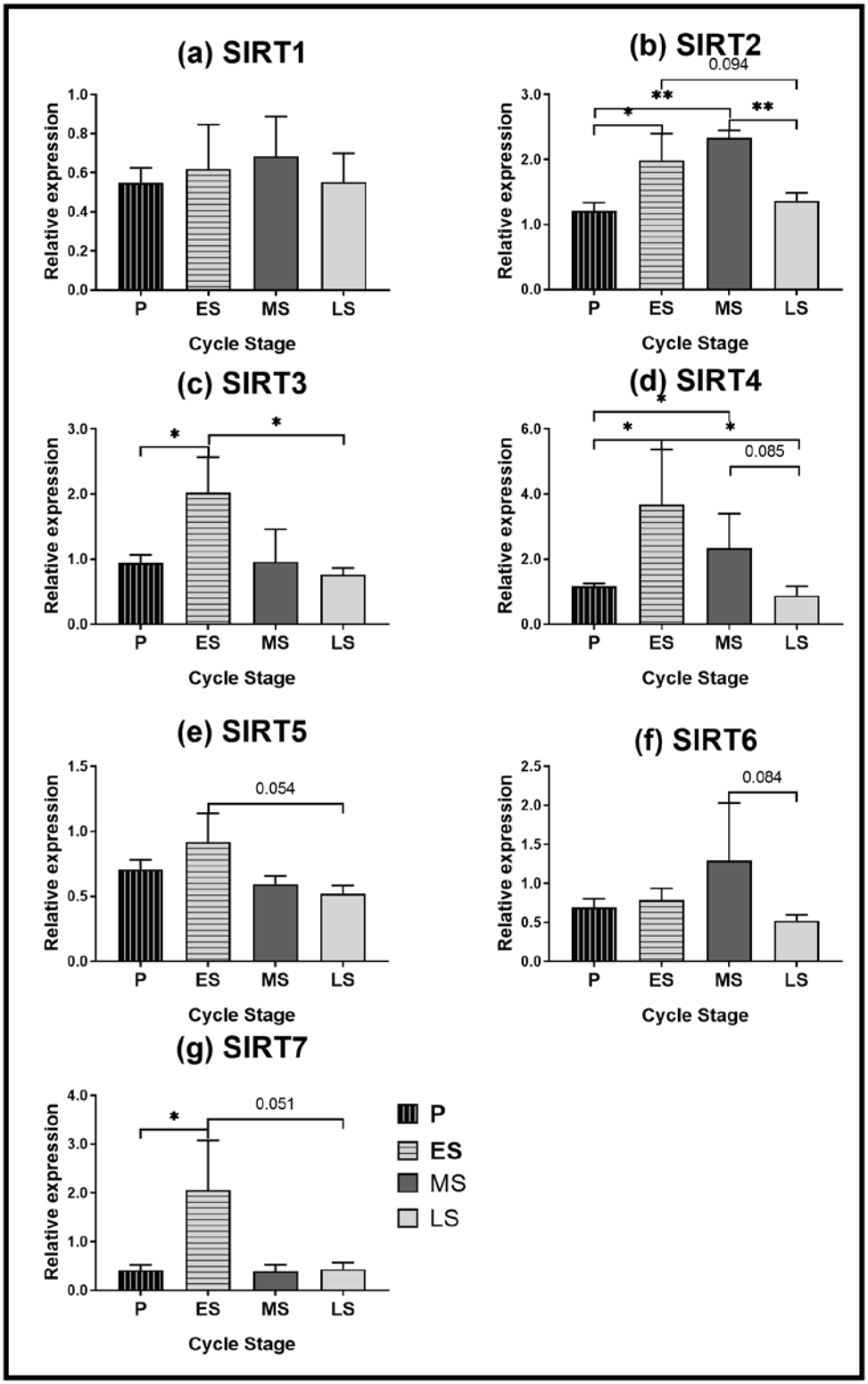

Figure 10: Relative mRNA expression of class 3 HDACs during menstrual cycle

Real time qPCR results for sirtuins in human endometrium during the menstrual cycle. 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

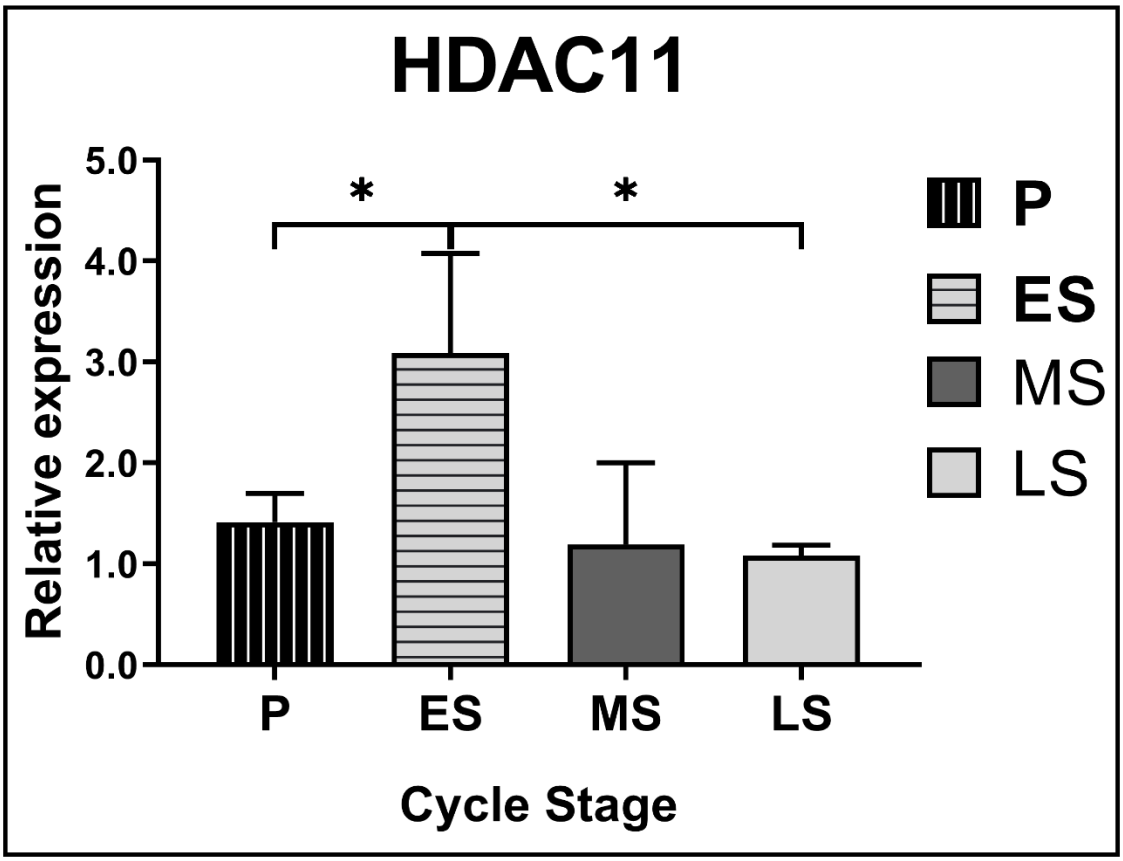

Figure 11: Relative mRNA expression of class 4 HDAC during menstrual cycle

Real time qPCR results for HDAC11 in human endometrium during the menstrual cycle. 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

3.2 Protein expression in endometrial tissue

HDAC protein expression was studied in endometrial tissue samples using fluorescent western-blot analysis. The samples were normalized against total protein stain. The results were analyzed using unpaired t-tests and plotted as Mean±SEM against the two menstrual cycle stages proliferative (n=7) and secretory phase (n=7). HDAC1, 2, and 3 proteins were constitutively expressed in both the proliferative and secretory phases. HDAC2 protein expression showed higher expression during proliferative phase nearing significance (P=0.097) (Figure 13). A similar trend was observed in HDAC3, with nearing significant increase in the proliferative phase (P=0.77) (Figure 14). HDAC8 and 4 displayed elevated protein expression during the secretory phase, but the difference was not significant (Figure 15 and 16). HDAC5 was significantly upregulated during the secretory phase with hardly any expression during the proliferative phase (*P<0.05)(Figure 17). It should also be noted that HDAC5 protein bands were observed slightly below 100 kDa, with consecutive bands observed below it. HDAC5 has three isoforms reported while 2 of the isoforms have 122kDa molecular weight one has a molecular weight of 112kDa, it is a possibility that isoform 2 of molecular weight 112kDa is observed during the experiment or a capsized version of protein was observed, either way, there is no proof yet to identify this. HDAC7 had no significant difference in protein expression between the two stages, but the band was observed slightly above 60kDa, the molecular weight of protein in 103kDa, but there are multiple isoforms of HDAC7, and the antibody used can detect multiple isoforms. The band size is possibly indicative that the isoform 10 (MW=66kDa) of HDAC7 was observed (Figure 18). HDAC9 was significantly upregulated during the secretory phase (**P<0.05) (Figure 19). It should be noted that the HDAC9 band was observed at 60kDa; according to Abcam, the antibody used can detect all the isoforms of HDAC9 except isoform 10 and 11. The isoform observed here may be either isoform 6 (MW=65kDa), 8 (MW=65kDa), or 9 (MW=60kDa).

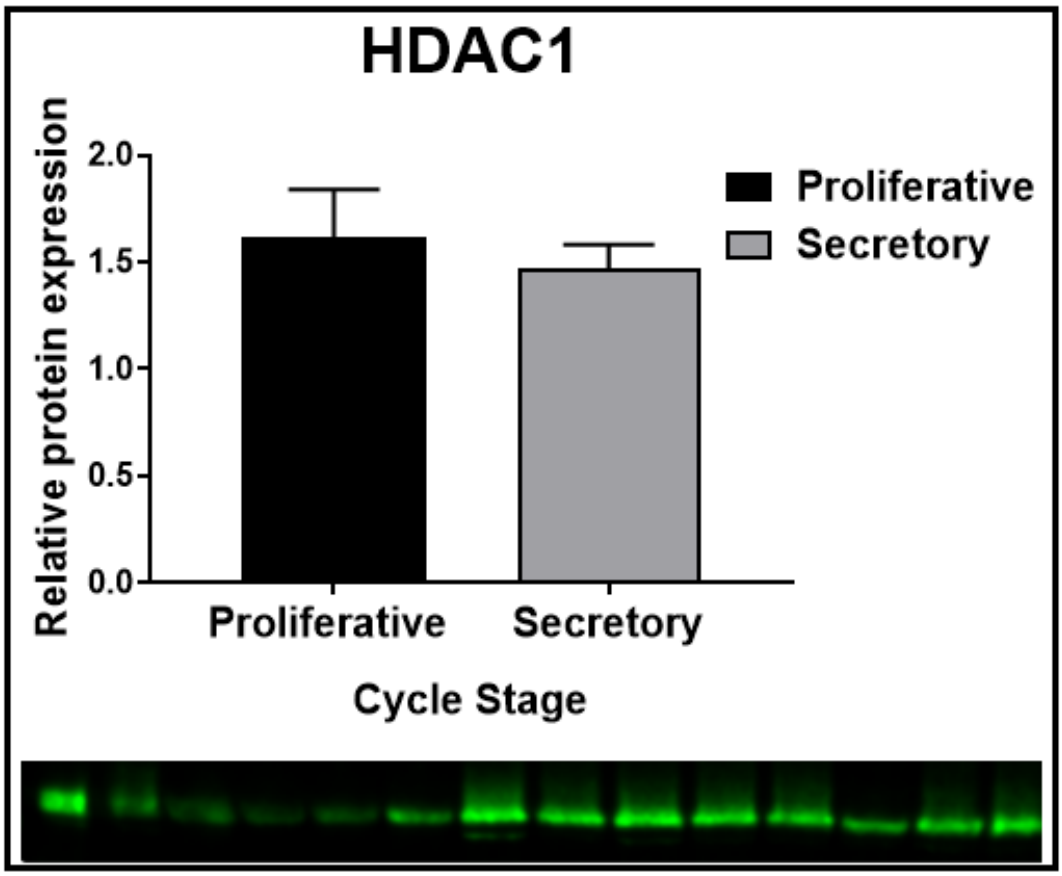

Figure 12: Changes in HDAC1 protein expression between proliferative and secretory phase

Relative protein expression levels normalized against total protein stain were plotted as Mean ± SEM against two cycle stages proliferative (n=7) and secretory phase (n=7). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$. The fluorescent bands indicate from left to right proliferative (n=7) and secretory (n=7).

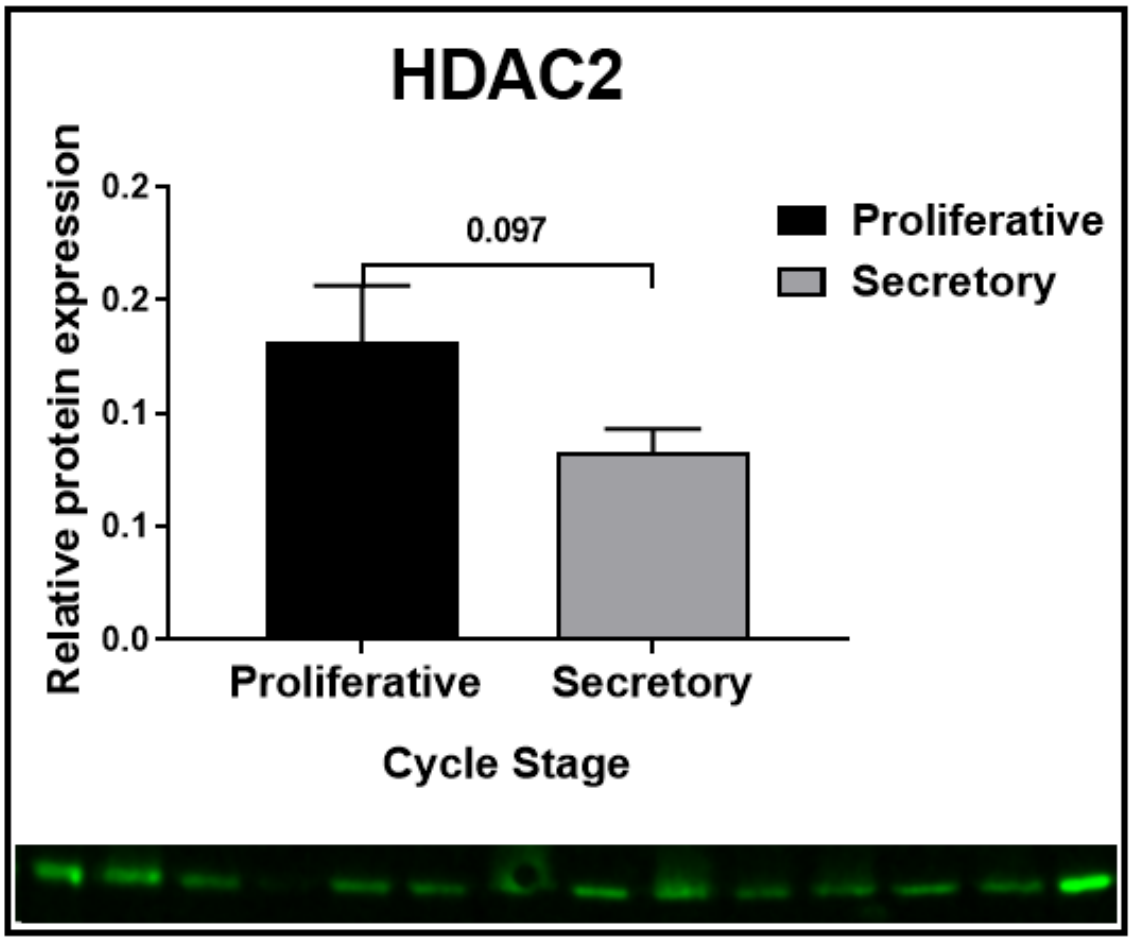

Figure 13: Changes in HDAC2 protein expression between proliferative and secretory phase

Relative protein expression levels normalized against total protein stain were plotted as Mean ± SEM against two cycle stages proliferative (n=7) and secretory phase (n=7). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$. The fluorescent bands indicate from left to right proliferative (n=7) and secretory (n=7).

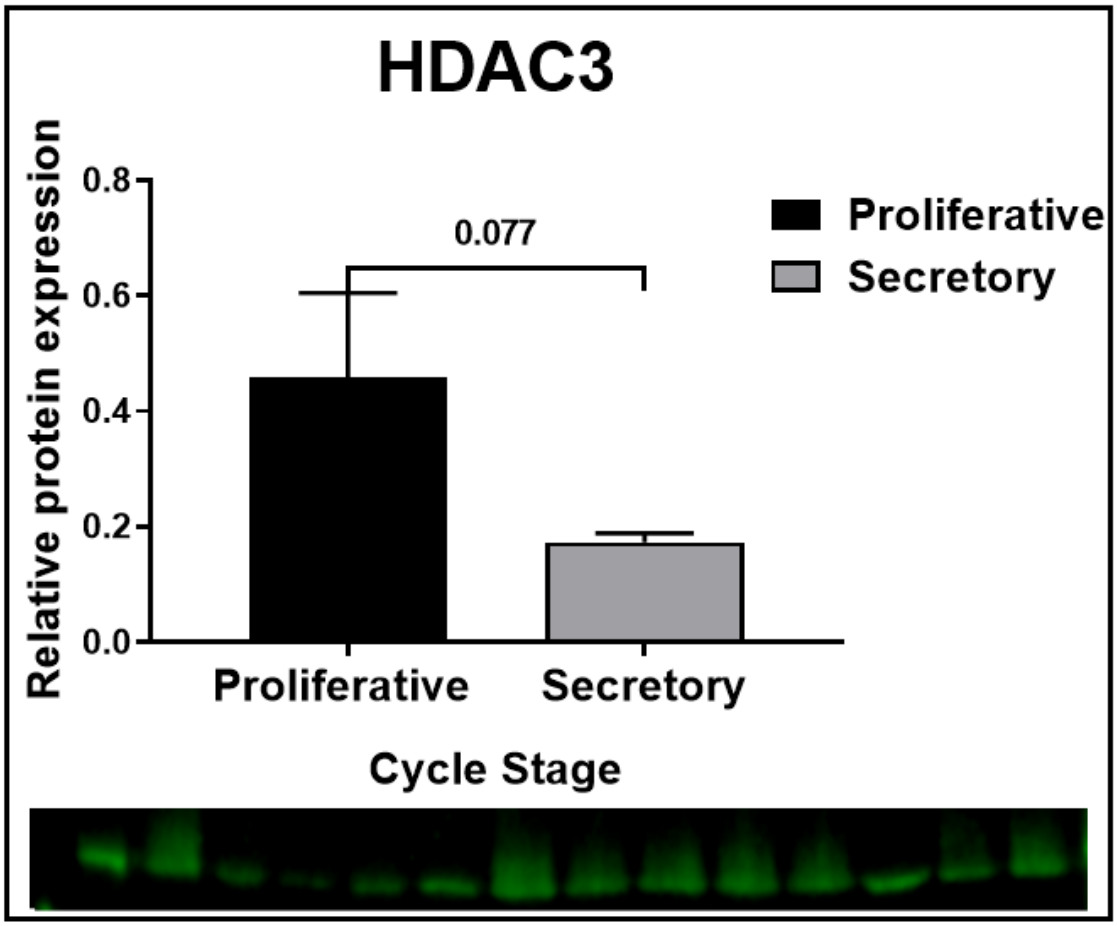

Figure 14: Changes in HDAC3 protein expression between proliferative and secretory phase

Relative protein expression levels normalized against total protein stain were plotted as Mean ± SEM against two cycle stages proliferative (n=7) and secretory phase (n=7). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$. The fluorescent bands indicate from left to right proliferative (n=7) and secretory (n=7).

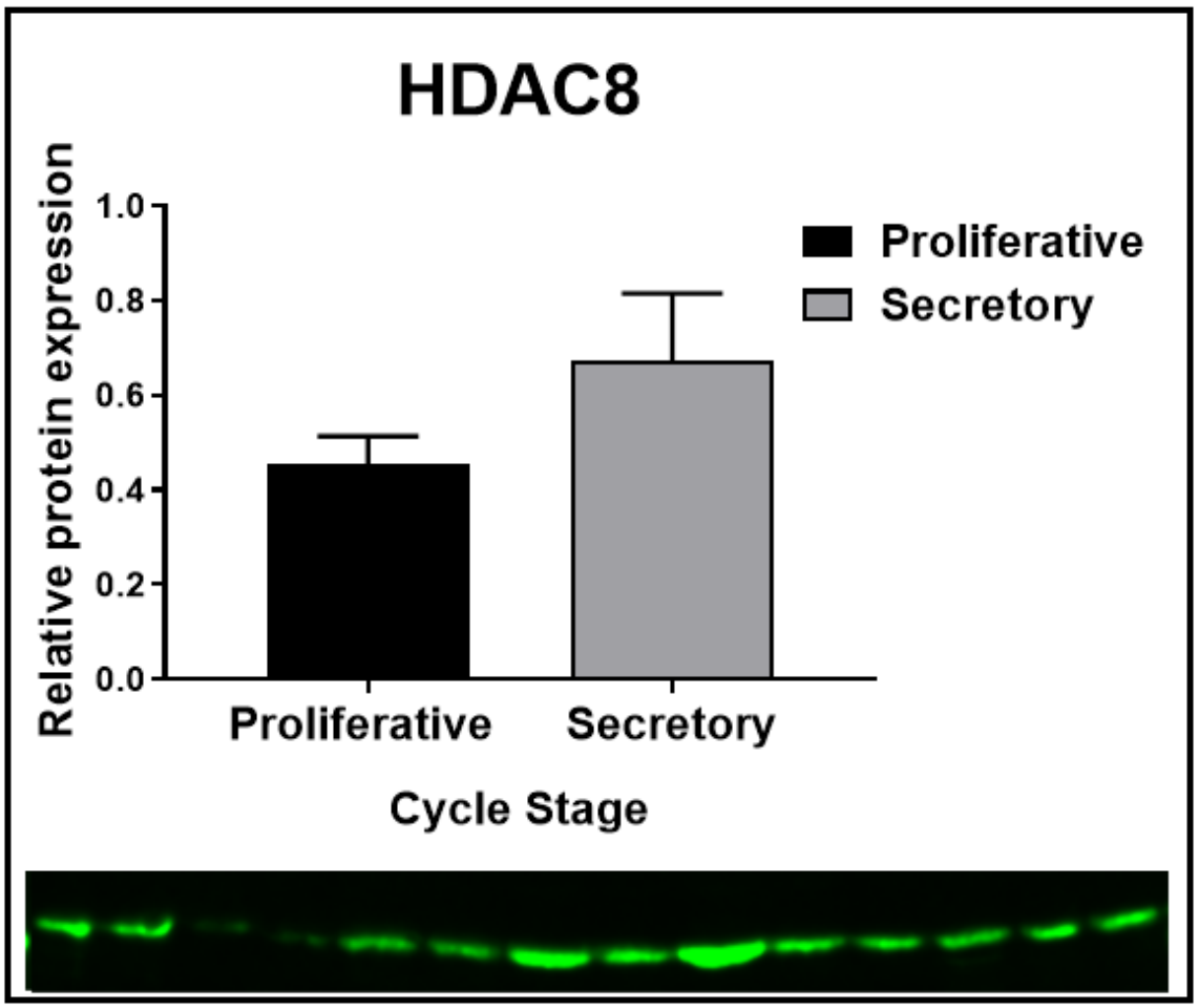

Figure 15: Changes in HDAC8 protein expression between proliferative and secretory phase

Relative protein expression levels normalized against total protein stain were plotted as Mean ± SEM against two cycle stages proliferative (n=7) and secretory phase (n=7). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$. The fluorescent bands indicate from left to right proliferative (n=7) and secretory (n=7).

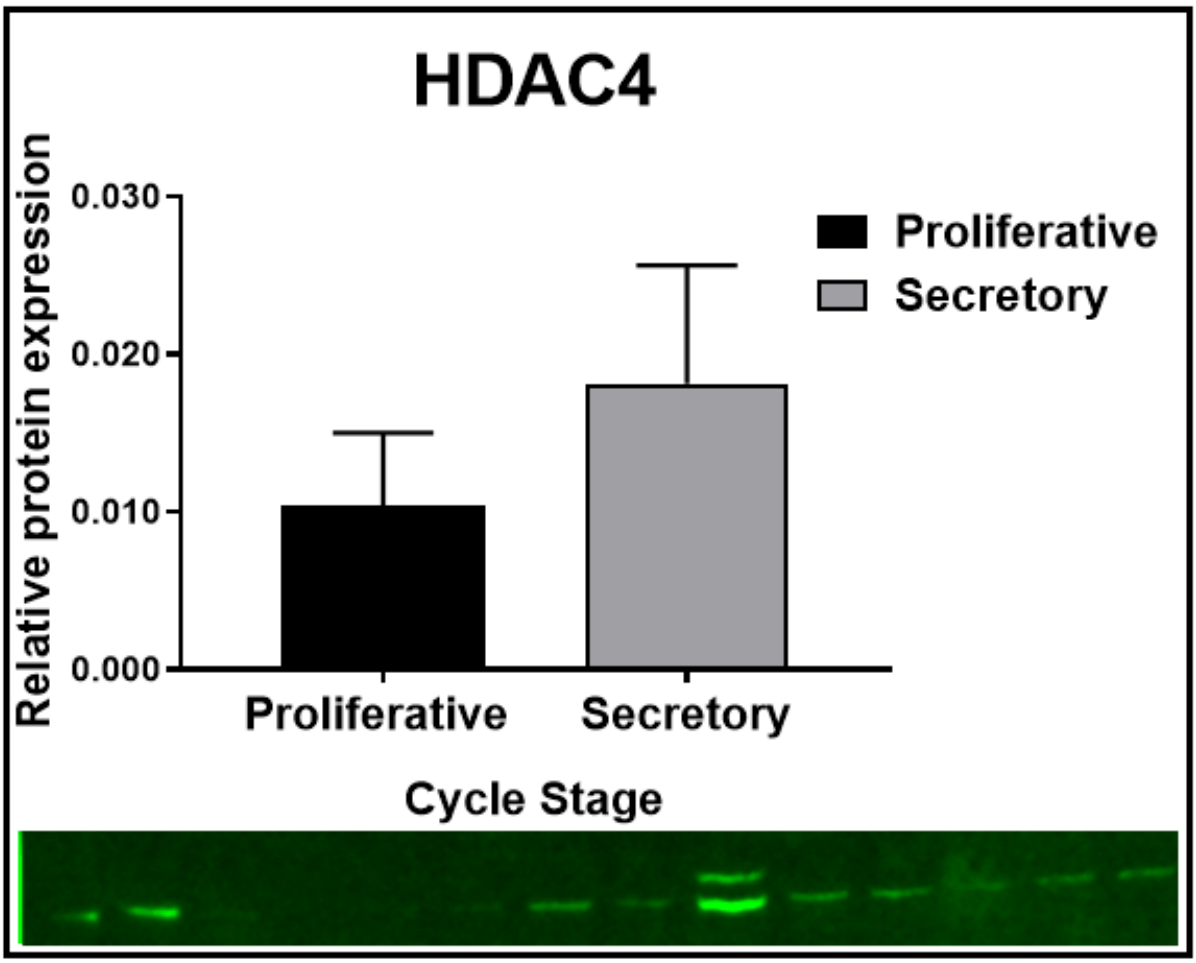

Figure 16: Changes in HDAC4 protein expression between proliferative and secretory phase

Relative protein expression levels normalized against total protein stain were plotted as Mean ± SEM against two cycle stages proliferative (n=7) and secretory phase (n=7). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. The fluorescent bands indicate from left to right proliferative (n=7) and secretory (n=7).

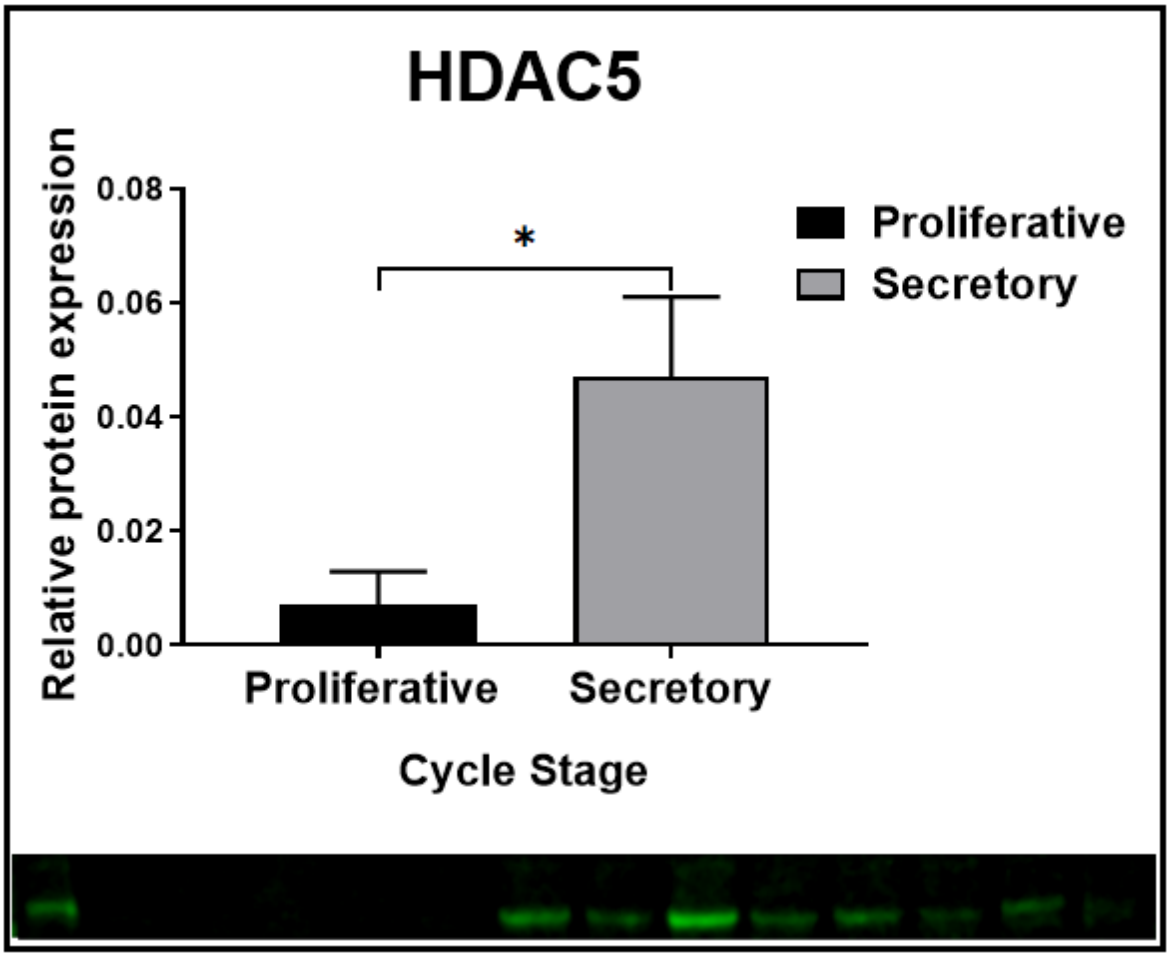

Figure 17: Changes in HDAC5 protein expression between proliferative and secretory phase

Relative protein expression levels normalized against total protein stain were plotted as Mean ± SEM against two cycle stages proliferative (n=7) and secretory phase (n=7). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$. The fluorescent bands indicate from left to right proliferative (n=7) and secretory (n=7).

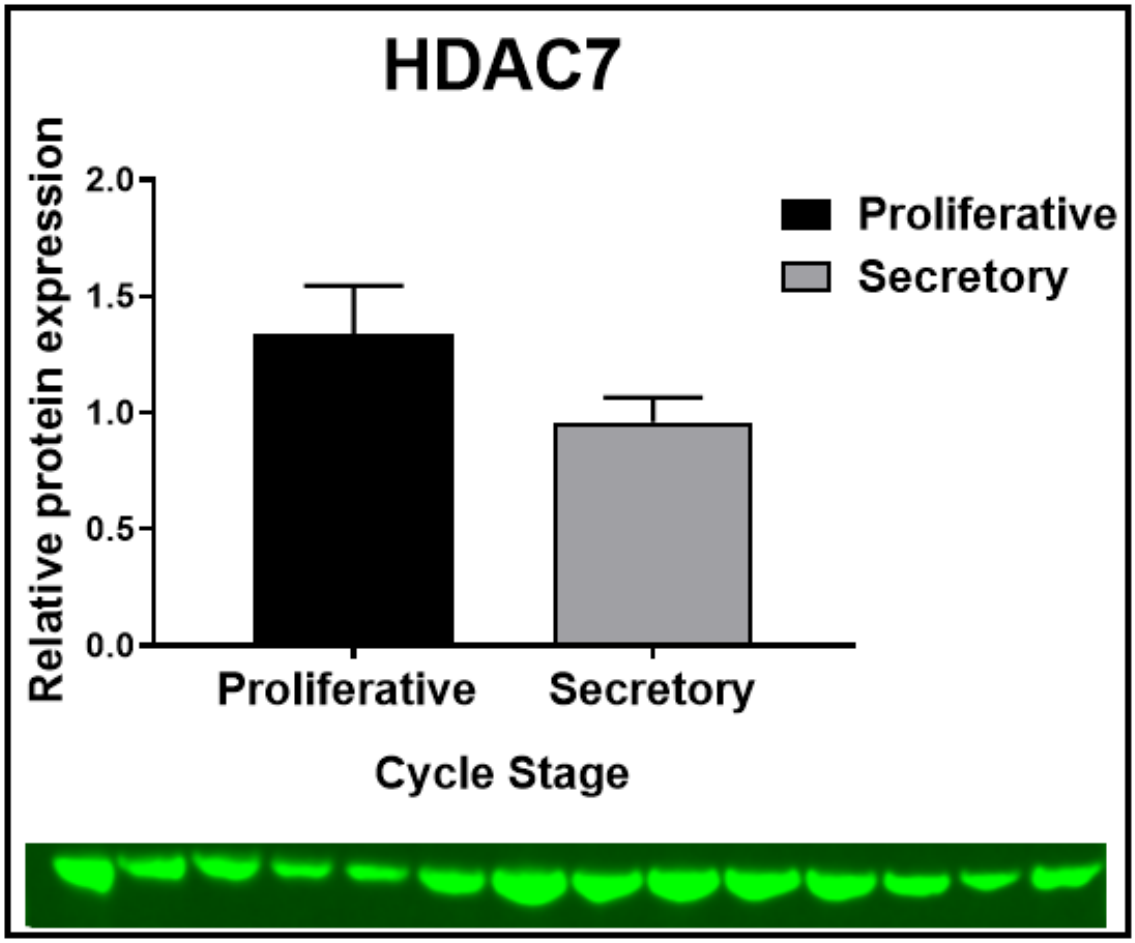

Figure 18: Changes in HDAC7 protein expression between proliferative and secretory phase

Relative protein expression levels normalized against total protein stain were plotted as Mean ± SEM against two cycle stages proliferative (n=7) and secretory phase (n=7). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$. The fluorescent bands indicate from left to right proliferative (n=7) and secretory (n=7).

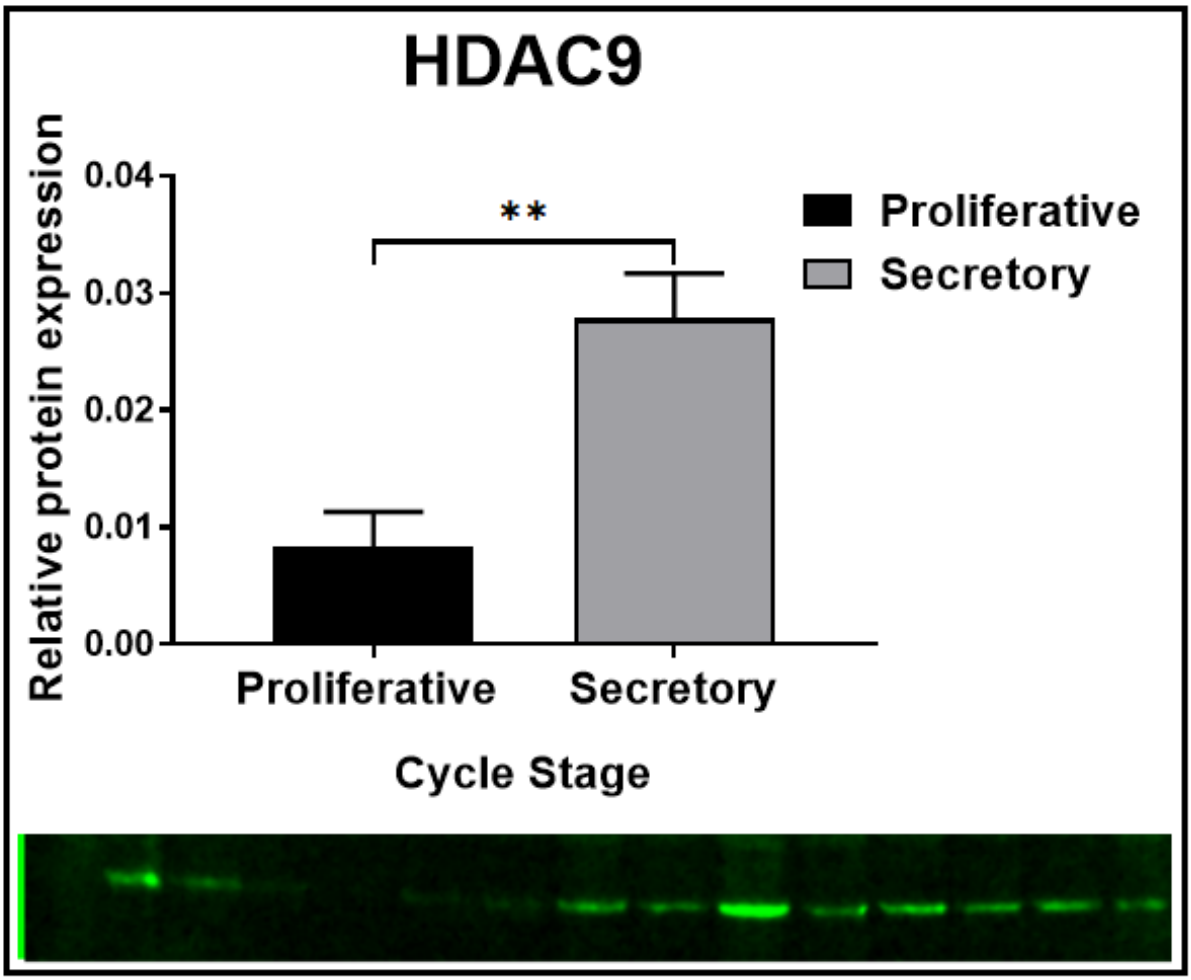

Figure 19: Changes in HDAC9 protein expression between proliferative and secretory phase

Relative protein expression levels normalized against total protein stain were plotted as Mean ± SEM against two cycle stages proliferative (n=7) and secretory phase (n=7). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$. The fluorescent bands indicate from left to right proliferative (n=7) and secretory (n=7).

3.3 Characterisation and Regulation of HDAC gene expression in endometrial cell lines

The effect of hormone treatment and HDAC inhibition by TSA on HDAC gene expression were studied in three different endometrial cell lines. AN3 and RL95-2 were used as a model for epithelial cells in the endometrium, while Human Endometrial Stromal cells (HESC) were used to observed changes in stromal cells. All the cell culture treatments were performed independently three times and analysed using 2-way ANOVA, and further significance was determined within each treatment group, and intervals using multiple comparisons uncorrected

Fisher's LSD, all tests were performed in GraphPad Prism 8.2.1 and SPSS. It should be noted that overall HDAC expression in HESCs was much lower than AN3 and RL95-2 cells with few HDACs having little or no expression.

3.3.1 HDAC gene expression in response to steroid hormones and TSA treatments in AN3 cells

AN3 cells showed significant differences in four HDAC genes within treatment groups (Figures 20-24). While there were no significant differences between treatment groups in gene expressions of HDACs 2, 3, and 8 (Figure 20 b-d), there was an overall significant difference seen in HDAC 1 mRNA expression throughout different time intervals (*P<0.05) and treatments (**P<0.01) and significant interaction between time and treatments were observed (**P<0.01). A significant increase in HDAC1 expression was seen in TSA+hormone treated cells after 24hrs as compared to the controls (**P<0.01) and cells treated with only estradiol (*P<0.05) (Figure 20 a).

In class 2a, HDAC3 showed a trend towards increased expression in response to estrogen and progesterone (E2+P4) between 24hrs to 72hrs (P=0.078) (Figure 21 b). There was a significant increase in HDAC3 expression of E2+P4 treated cells between 24hrs to 48hrs (*P<0.05) while a significant decline was observed in E2+P4 treated cells after 48 hrs (*P<0.05) (Figure 21 c). Little to no HDAC9 gene expression was observed in AN3 cells.

HDAC6 gene expression reduced significantly after 24hrs of treatment with E2+P4 in conjunction with TSA compared to the control (*P<0.05) (Figure 22 a). HDAC 6 also shared a similar expression trend with HDAC7, showing a significant increase in E2+P4 treated cells from 24hrs to 48hrs and then subsequent significant decline in expression after 48hrs (Figure 22 a). HDAC10 showed no significant changes in response to any treatments (Figure 22 b).

Only expressions of SIRT2 and 7 showed significant changes in response to treatment among the seven sirtuins. SIRT2 showed a significant increase in gene expression in TSA + steroid hormone treated group after 24hrs, compared to controls (*P<0.05), as well as E2 treated cells (*P<0.05) (Figure 23 b). A subsequent decline was observed in TSA + steroid hormone treated cells in each 24hr interval after that (Figure 16 b). SIRT5 gene expression was significantly high in E2+P4 treated cells at 72hrs compared to its TSA + steroid hormone treated counterparts (*P<0.05) (Figure 23 e). SIRT7 showed increased expression after 48hrs of treatment with E2+P4 as compared to controls (*P<0.05), it also showed the significantly highest expression at this interval compared to 24hrs and 72hrs of E2+P4 treatment (*P<0.05) (Figure 23 g). HDAC11 showed a trend toward an increase in E2+P4 treated cells at 72hrs as compared to E2 treated cells for 24hrs(P=0.076) (Figure 24).

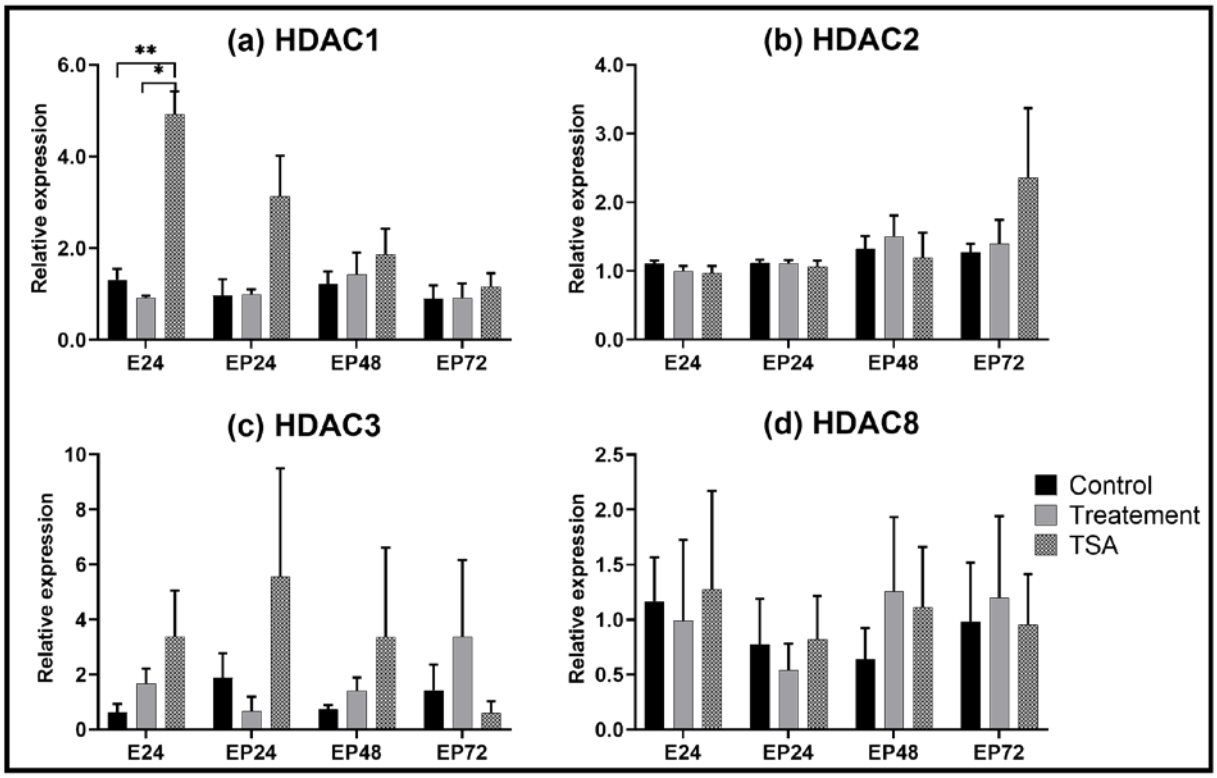

Figure 20: Relative mRNA expression of class 1 HDACs in response to steroid hormone treatment and TSA in AN3

Real time qPCR results for class 1 HDACs (1, 2, 3 and 8) in AN3 control, hormone treatment and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against tow housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$.

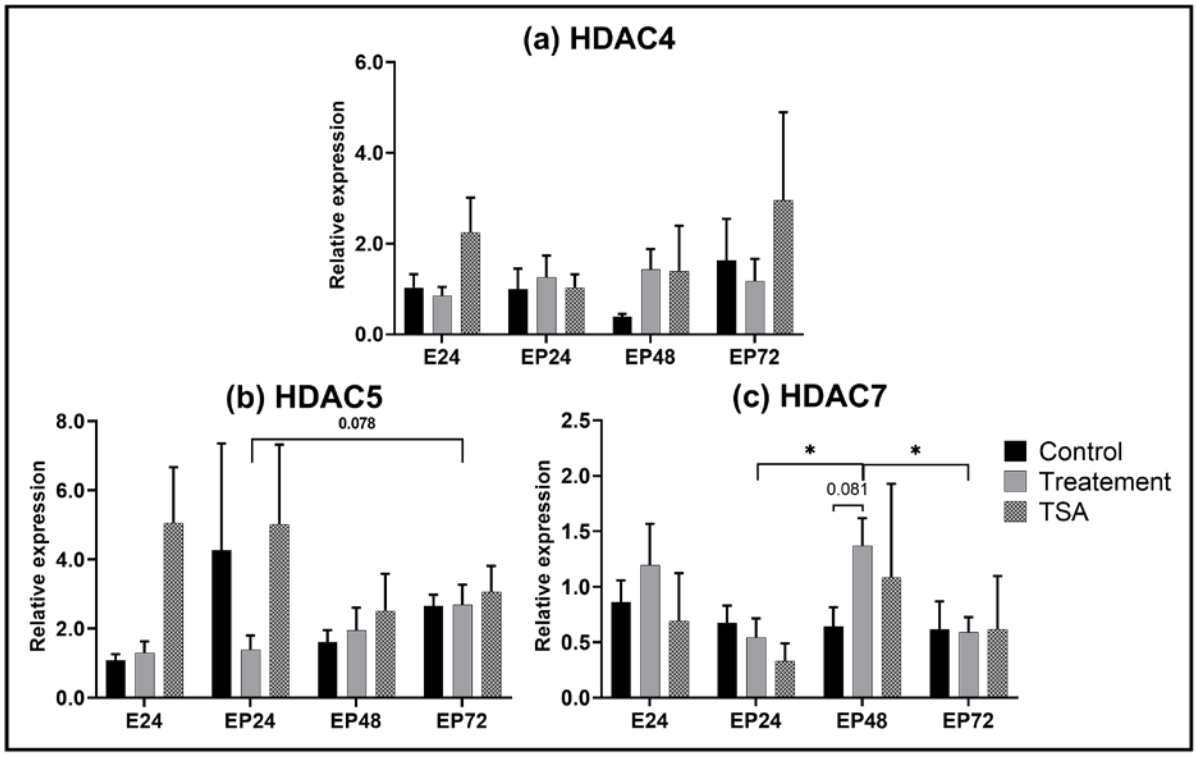

Figure 21: Relative mRNA expression of class 2a HDACs in response to steroid hormone treatment and TSA in AN3

Real time qPCR results for class 2a HDACs (4, 5, and 7) in AN3 control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against two housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * p≤0.05, ** p ≤0.01, ***p ≤0.001.

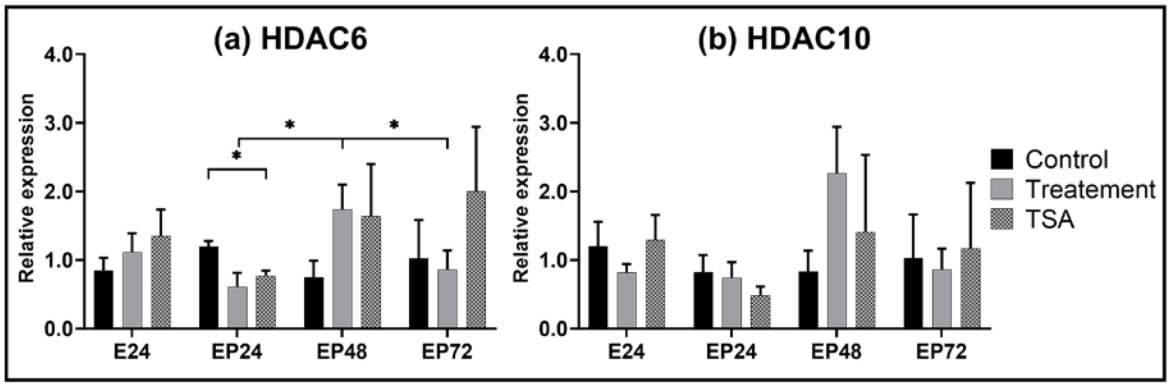

Figure 22: Relative mRNA expression of class 2b HDACs in response to steroid hormone treatment and TSA in AN3

Real time qPCR results for class 2b HDACs (6 and 10) in AN3 control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against two housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * p≤0.05, ** p ≤0.01, ***p ≤0.001.

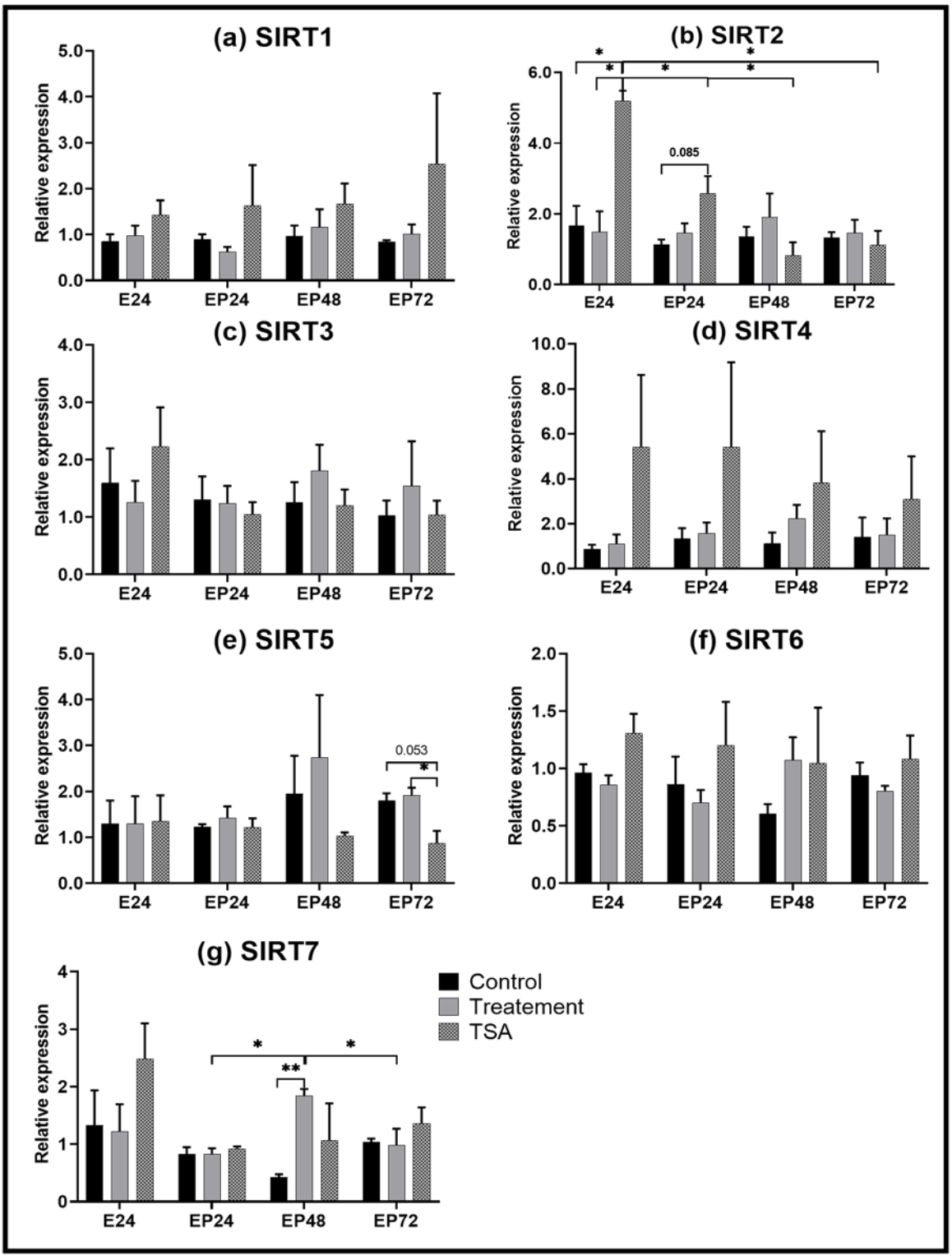

Figure 23: Relative mRNA expression of class 3 HDACs in response to steroid hormone treatment and TSA in AN3

Real time qPCR results for sirtuins (1 to 7) in AN3 control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against two housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

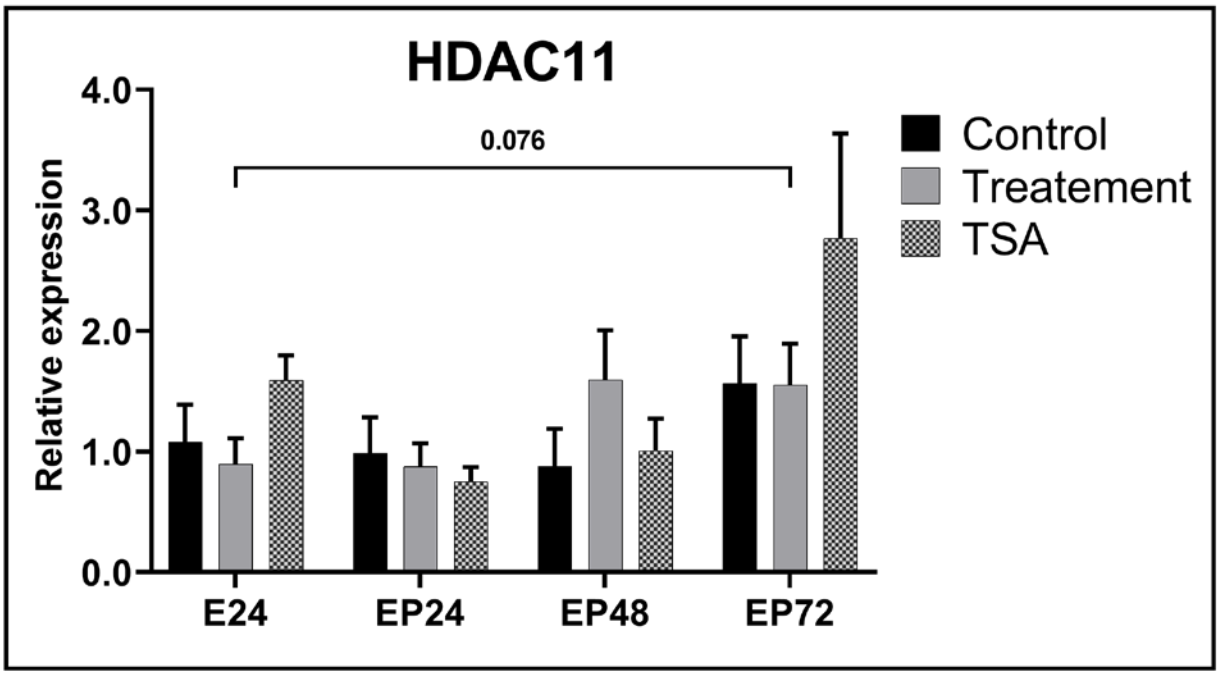

Figure 24: Relative mRNA expression of class 4 HDAC in response to steroid hormone treatment and TSA in AN3

Real time qPCR results for HDAC11 in AN3 control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against two housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.3.2 HDAC gene expression in response to steroid hormones and TSA treatments in RL95-2 cells

A significant increase in HDAC1 expression level was seen in TSA+ E2 + P4 treated cells from 24hrs to 72hrs (*P<0.05) (Figure 25 a), while HDAC2 expression levels were significantly high at initial E2 treatment of 24hrs compared to 72hrs of treatment with E2+P4 (*P<0.05) (Figure 25 b). HDAC8 showed an increase in expression trend between cells treated with E2 for 24hrs and cells treated with E2+P4 after 72hrs, but the p-value was not significant (P=0.060). HDAC3 expression was very low in RL95-2 cells in response to all treatments.

Significant changes were observed in class 2a HDACs 5, 7, and 9. A trend toward an increased expression was observed in HDAC4 from E2+P4 treated cells for 24hrs to the E2+P4 cells at 72hrs, but the p-value was not significant (P=0.084) (Figure 26). A significant increase in HDAC5 expression levels was observed in E2+P4 treated cells after 48hrs compared to cells treated with E2 for 24hrs and E2+P4 for 24hrs (*P<0.05), there was also an overall significant expression change over different time intervals (*P<0.05) (Figure 26 b). HDAC7 expression was significantly increased after treatment with TSA and estrogen for 24hrs as compared to its controls and cells treated with estrogen (*P<0.05), while a trend towards an increase was seen in cells treated with steroid hormones over time but it was not significant (Figure 26 c). HDAC9 levels were really low in the RL95-2 cells and an even further significant reduction in expression was seen in E2+P4 treated cells at 72hrs interval as compared to their controls (*P<0.05).

HDAC6 showed an overall significant change over time (*P<0.05), while a significant increase was seen in expression of HDAC6 in cells with time in response to TSA+ E2 +P4 at 24hrs to cells treated with TSA+ E2 +P4 at 72hrs (*P<0.05) (Figure 27 a). A similar trend was observed in HDAC10 expression levels with a significant upregulation observed in cells treated with TSA +E2 over 24hrs in comparison to controls (*P<0.05). A trend towards reduction in expression level was observed in TSA+ E2+P4 treated cells after 24hrs of treatment with a subsequent increase every 24hr interval, but the p-values were not significant (Figure 27 b).

Sirtuins showed varied expressions in response to steroid hormone treatments, with significant changes observed in SIRT4, 5 and 6. A significant reduction in SIRT4 was observed in cells treated with TSA+E2 as compared to controls (*P<0.05) (Figure 28 d). An overall significant difference was observed in SIRT5 (*P<0.05) expression levels throughout the treatment with respect to time, with a trend towards an increased expression observed after 48 hrs of treatment

with E2+P4 but there were no significant changes within groups (Figure 28 e). An increase in SIRT6 expression was seen in cells treated with TSA+E2 after 24hrs in comparison to control (*P<0.05), whereas significant reduction seen in treatment with TSA+E2+P4 after 48hrs of treatment compared to TSA+E2+P4 after 24hrs (*P<0.05) (Figure 28 f). A trend towards a reduced expression was seen after 24hrs of TSA+E2+P4 treatment from the initial peak after 24hrs of treatment with TSA+E2 (P=0.066) (Figure 21 g).

An overall significant change was observed in HDAC11 with time (**P<0.01) and treatment (***P<0.001), a significant increase was seen in controls for EP48 hrs as compared to initial control of E24 (*P<0.05) (Figure 29).

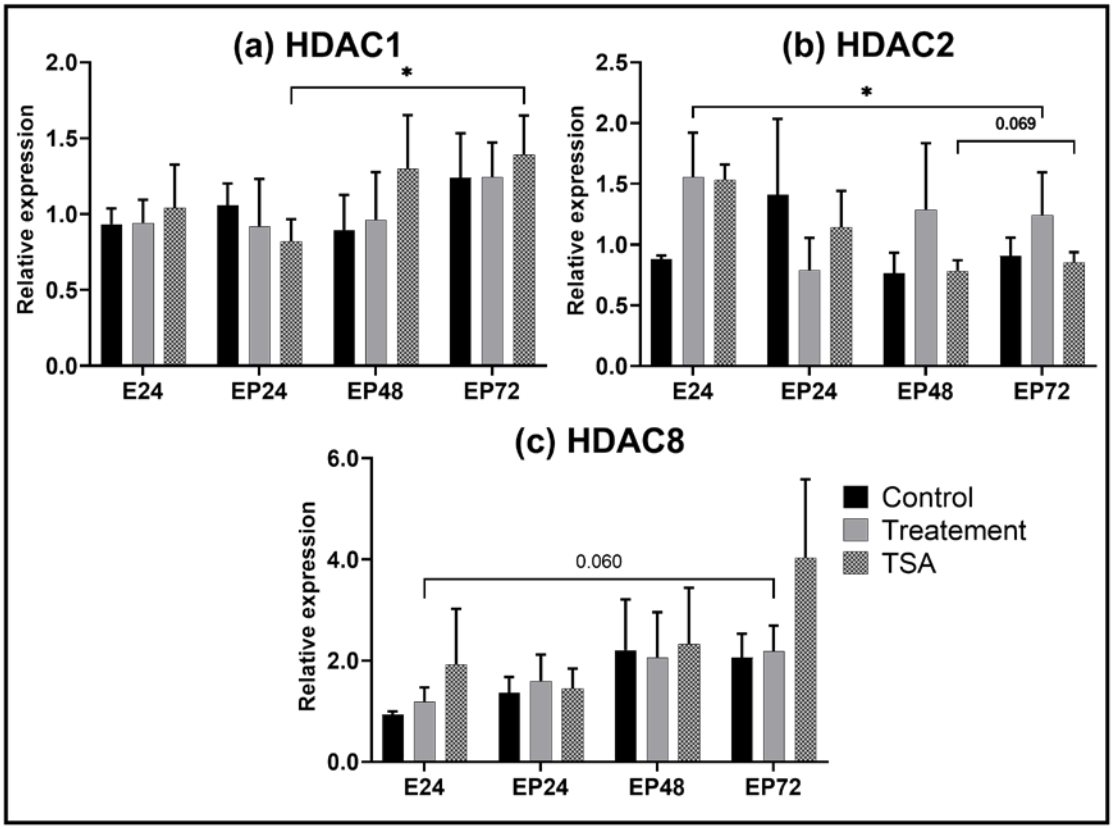

Figure 25: Relative mRNA expression of class 1 HDACs in response to steroid hormone treatment and TSA in RL95-2

Real time qPCR results for class 1 HDACs (1, 2, and 8) in RL95-2 control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against tow housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * p≤0.05, ** p ≤0.01, ***p ≤0.001.

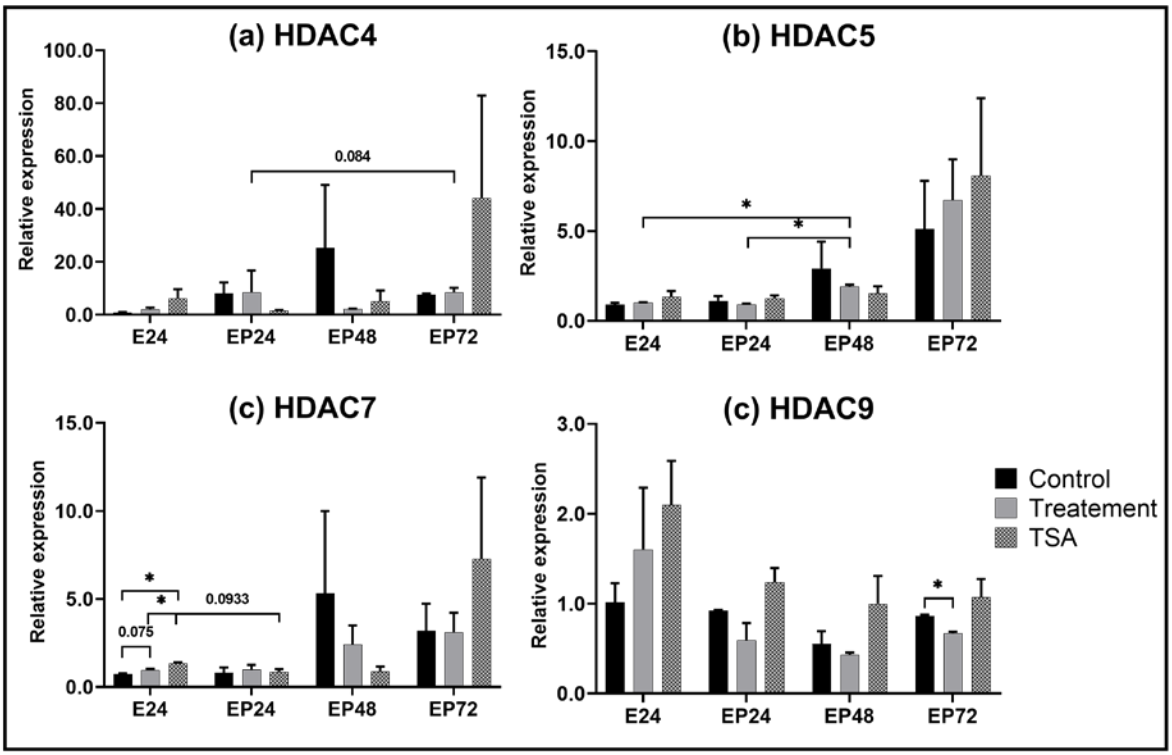

Figure 26: Relative mRNA expression of class 2a HDACs in response to steroid hormone treatment and TSA in RL95-2

Real time qPCR results for class 2a HDACs (4, 5, 7, and 9) in RL95-2 control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against tow housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * p≤0.05, ** p ≤0.01, ***p ≤0.001.

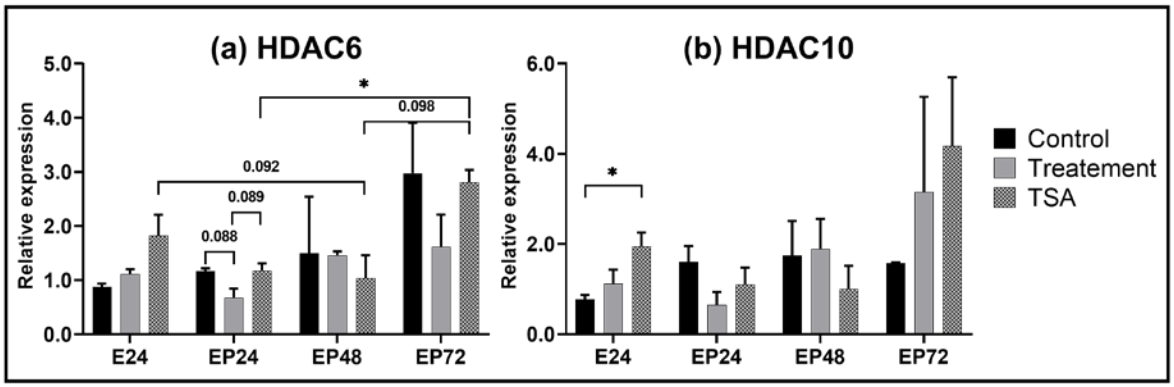

Figure 27: Relative mRNA expression of class 2b HDACs in response to steroid hormone treatment and TSA in RL95-2

Real time qPCR results for class 2b HDACs (6 and 10) in RL95-2 control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against tow housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * p≤0.05, ** p ≤0.01, ***p ≤0.001.

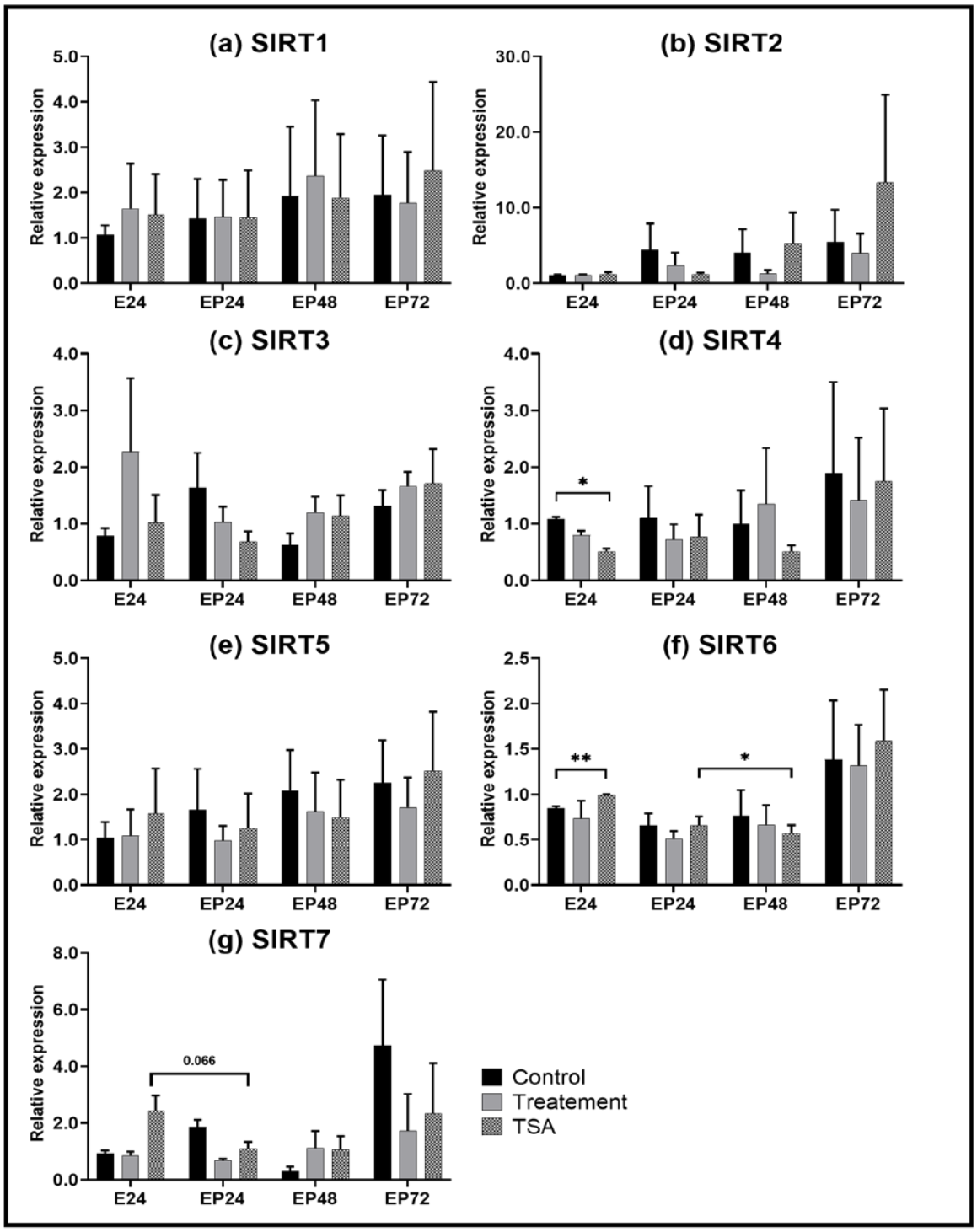

Figure 28: Relative mRNA expression of class 3 HDACs in response to steroid hormone treatment and TSA in RL95-2

Real time qPCR results for sirtuins (1, 2, 3, 4, 5, 6 and 7) in RL95-2 control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against tow housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * p≤0.05, ** p ≤0.01, ***p ≤0.001.

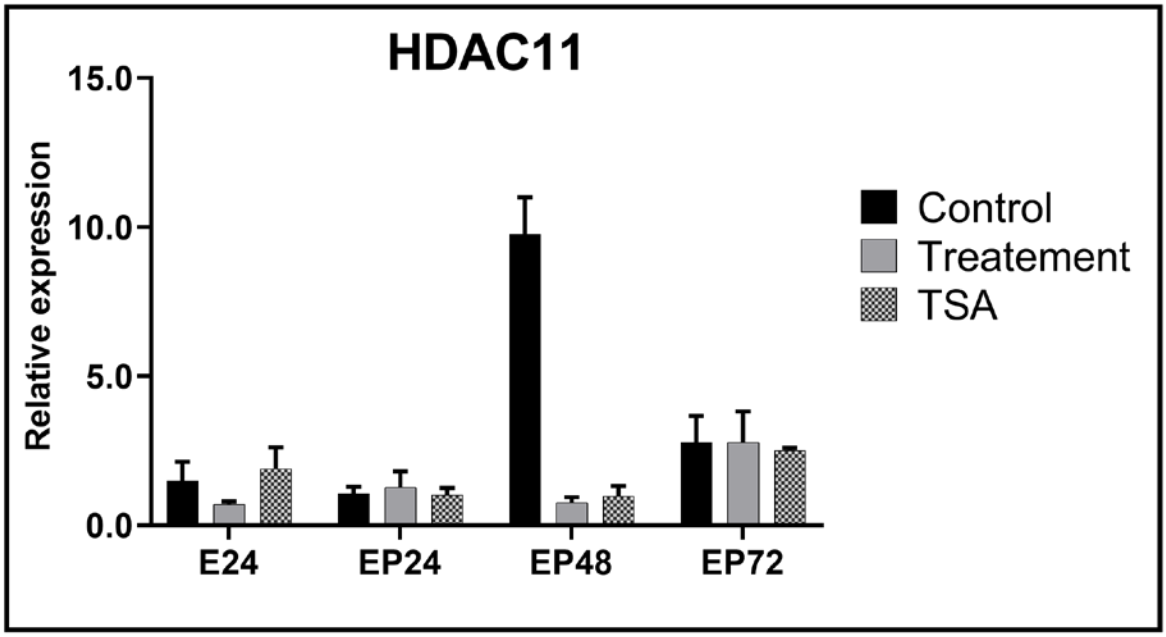

Figure 29: Relative mRNA expression of class 4 HDACs in response to steroid hormone treatment and TSA in RL95-2

Real time qPCR results for HDAC11 in RL95-2 control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against tow housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$.

3.3.3 HDAC gene expression in response to steroid hormones and TSA treatments in HESC cells

HESC had low amounts of all HDAC expressions throughout the treatment groups except HDAC1 and HDAC2. HDAC3, class 2 HDACs (4, 5, 6, 7, 9, and 10), SIRTs (4, 5, and 7), and HDAC10 showed very little to no expression in all three replicates, many ct values were observed beyond 35 hence was not reliable to analyse. HDAC 1 showed a significant reduction in expression levels after 48hrs of treatment with E2+P4 as compared to cells treated with E2+P4 for 24hrs (*$P<0.05$). HDACs 2 and 8 did not observe any significant changes throughout the menstrual cycle (Figure 30 a-c).

Out of all the sirtuins, SIRT 1 was most stably expressed in the HESC cells, although no significant differences are observed in any of the sirtuin expression levels (Figure 31). It should be noted that

overall HDAC expression in HESCs was much lower than AN3 and RL95-2 cells with few HDACs having little or no expression.

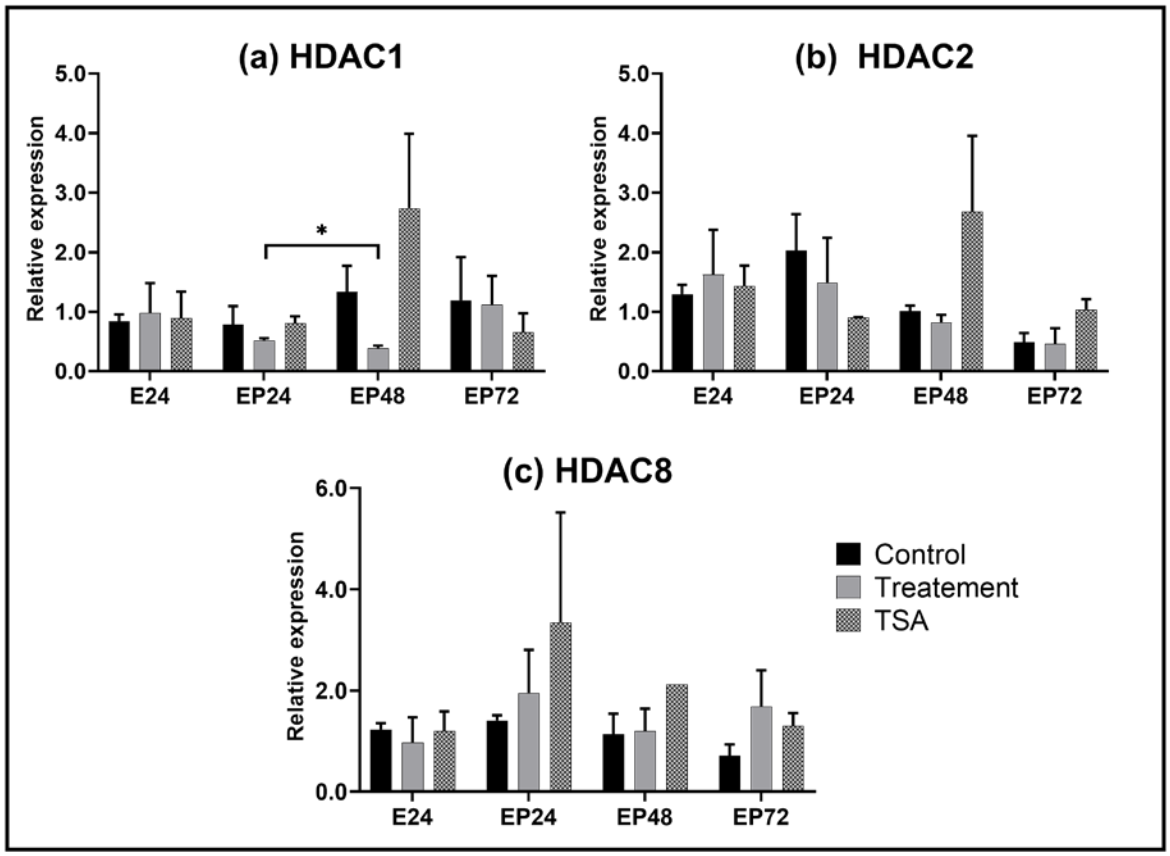

Figure 30: Relative mRNA expression of class 1 HDACs in response to steroid hormone treatment and TSA in HESC

Real time qPCR results for class 1 HDACs (1, 2 and 8) in HESC control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against tow housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

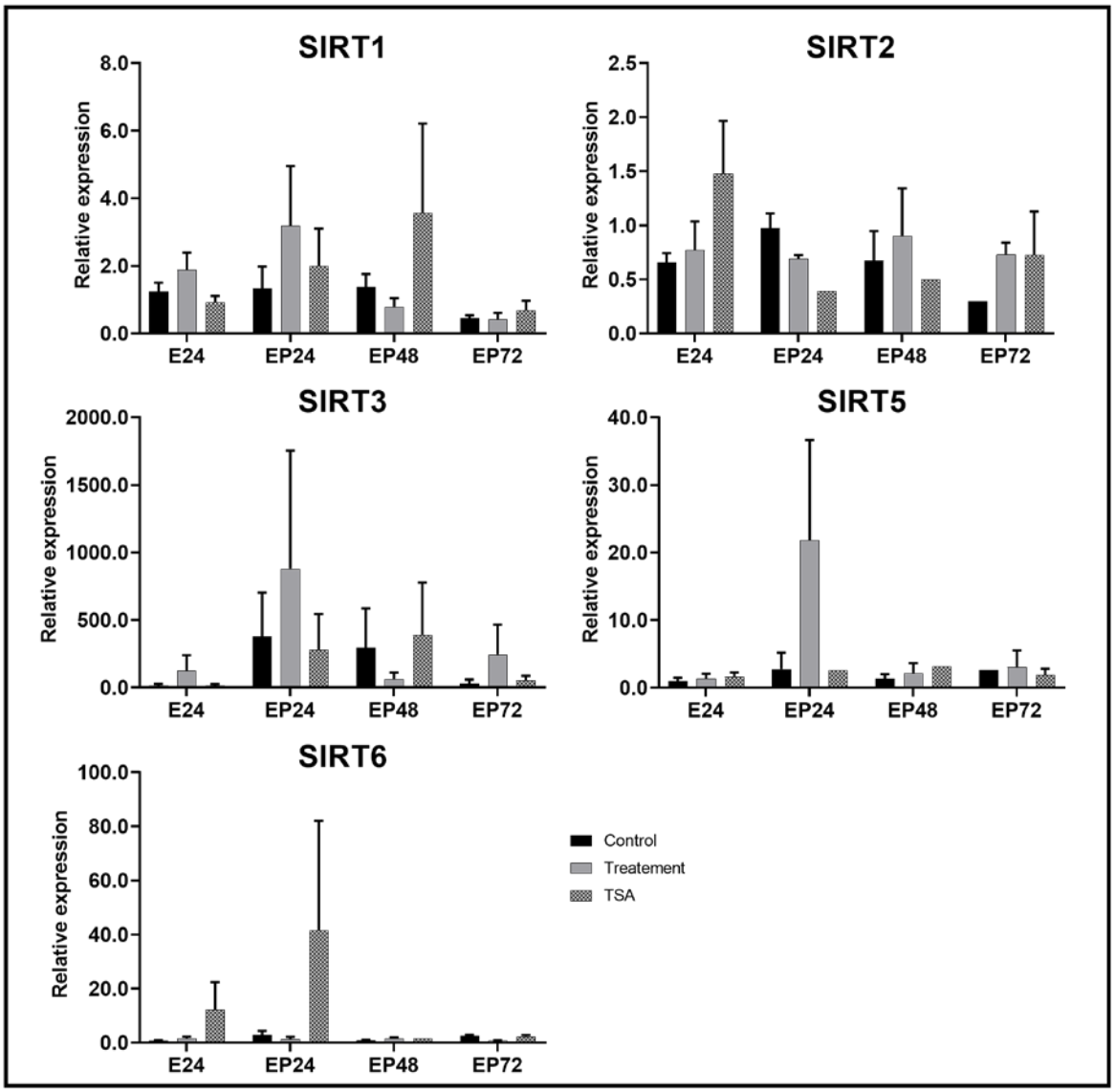

Figure 31:Relative mRNA expression of class 3 HDACs in response to steroid hormone treatment and TSA in HESC

Real time qPCR results for sirtuins (1,2, 3, 5 and 6) in HESC control, hormone treatment, and hormone with TSA treated cells harvested at 24hr intervals. The Y-axis shows the relative mRNA expression normalized against tow housekeeping genes YWHAZ and RPL13A represented as Mean ± SEM (n=3). The X-axis shows the different treatment intervals: estradiol (0.01µM) for 24hrs (E24), estradiol (0.01µM) and progesterone (1µM) for 24hrs (EP24hrs), estradiol (0.01µM) and progesterone (1µM) for 48hrs (EP48) and estradiol (0.01µM) and progesterone (1µM) for 72hrs (EP72hrs). Statistical significance is given as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

4 Discussion

This master's project aimed to characterize gene and protein expressions of histone deacetylases (HDACs) in normal endometrium during the menstrual cycle and determine whether they are regulated by steroid hormones in endometrial stromal and epithelial cells. The role of HDACs and their inhibitors have been investigated in several endometrial pathologies (139, 215, 216, 220, 226). However, there are very few studies investigating the role of individual HDACs in normal endometrium during the menstrual cycle stages. A previous study from our lab demonstrated temporal changes in global histone acetylation levels throughout the menstrual cycle. It appeared to follow a trend of increase in acetylation after menstruation with subsequent decline until ovulation with acetylation level lowest during and around the time of ovulation (232). Most of the HDAC gene expression levels in tissue follow a pattern complementary to the global histone acetylation levels observed in the previous study. Endometrial tissue broadly contains two kinds of the cell population, epithelial and stromal cells. The expression levels in the tissue give us a comprehensive outline of what happens in the tissue. To understand the individual functions of HDACs and the influence of steroid hormones on their expression levels in the endometrium, I further worked on the cell models for epithelial and stromal cells. Overall, the data imply that HDAC expressions vary widely in cell lines, in response to the same steroid treatments. The treatment with HDAC inhibitor TSA often has a cell line specific effect on HDAC expressions.

Further investigation is required to understand the individual functions of these HDACs in different cellular populations, and how they interact and regulate gene expression in the endometrium. However, the results of this research are discussed below.

4.1 HDAC expression and histone acetylation during the menstrual cycle

Relative HDAC gene expression in endometrial tissue follows a pattern, with most of the HDACs being upregulated during the early secretory and the mid secretory phase, with comparatively low expression during the late secretory and proliferative phase. During the early secretory phase, most of the proliferative pathways are no longer required, and the secretory transformations have not yet begun. A significant increase is observed during this phase in HDAC1, HDAC8, HDAC5, HDAC7, SIRT3, SIRT4, SIRT7, and HDAC11, while SIRT5 reached near significance (Figure 32). Since HDACs are usually associated with gene silencing, this trend is suggestive of their involvement in stopping the proliferation-related gene expression facilitating a switch from proliferative to secretory endometrium. It's been observed previously that the global histone acetylation patterns follow a similar trend with a progressive increase in global histone acetylation levels after menstruation and then decreasing as the cycle progresses towards ovulation, which is followed by again increase in acetylation post ovulation (232). While HDAC1 gene expression is significantly upregulated during the early secretory phase, the HDAC2 expression level was highest during the mid secretory phase. HDAC 1 and 2 are usually involved in cellular functions such as proliferation and apoptosis (114, 119), they play a critical role in hormone dependent gene expression (120). HDACs 1 and 2 can form complexes with each other, which allows them to function both together or individually and complimenting each other (121-123). Both protein and mRNA expression levels are quite high for these two HDACs. While there was no significant difference seen in protein expression between the proliferative and secretory phases in HDAC1, HDAC2 was highly upregulated during the proliferative phase. HDAC2 gene expression, although peaks during the mid secretory phase it remains consistently high during the proliferative and early secretory phase. HDAC2 is thought to play a crucial role in cell proliferation and cell cycle control by suppressing the expression of p21 and p57 and assisting in G1 to S phase transition during the

cell cycle (233). This could be an essential factor for its stable expression during the proliferative phase. It should be noted that HDAC3 had little to no gene expression throughout the cycle stages, while HDAC2 was very highly expressed, which is consistent with a previous study (128). On the other hand, significant expression of HDAC3 protein is seen in the tissue biopsies; this could be an indicator of the longer half-life of the protein while mRNA turnover is quick. Studies have shown a crucial role of HDAC3 during implantation due to its involvement in transcription activation of *COL1A1* and *COL1A2* (collagenase) genes in humans and *col1a1* and *col1a2* genes in mice (211), which are involved in endometrial remodelling and trophoblast invasion during implantation (206). HDAC8 mRNA expression was significantly high during the early secretory phase, its protein expression was seen to be high during the secretory phase, but without any statistical significance. HDAC4 also followed a similar pattern in both the gene and protein expression studies with no significant difference throughout the cycle. The gene expression levels of HDAC5 was high in both early and mid secretory phase and its protein expression followed suite with significantly high expression during the secretory phase with hardly any expression during the proliferative phase. Studies have shown that HDAC5 is weekly expressed in endometrial carcinomas, this could be indicative of HDAC5 not being involved in proliferative pathways in the endometrium (139).

HDAC9 also showed significantly high expression during the mid secretory phase with very low expression levels throughout the other cycle stages, which was seen in the protein expression as well. It is during the mid secretory phase that the implantation window in the menstrual cycle is observed, and HDAC9 is known to suppress TIMP3, promoting trophoblast invasion, and promoting implantation of the blastocyst (234). A significant peak in HDAC10 gene expression was also seen during the mid secretory phase sharing a similar, non-significant trend with HDAC6. HDAC10 levels slightly dropped during the late secretory phase but had a nearly significant expression as compared to the proliferative phase. HDAC 10 is associated

with DNA mismatch repair, and increased expression of HDAC10 is linked to good prognosis in cancers. There are no precise functions of HDAC10 explored so far, but a study has shown that an increased level of HDAC10 can help suppress cervical cancer metastasis by inhibiting Matrix Metalloproteinase (MMP) 2 and 9 (235). While MMP2 is crucial for trophoblast invasion, decidual cells also produce inhibitors of MMPs to protect complete degradation of the endometrium, maintaining a fine-tune during the mid secretory phase (206, 208, 209). Increased expression of HDAC10 at the mid secretory phase could be associated with the required inhibition of MMPs during this phase, but further investigation is required.

All the sirtuins followed unique expression patterns with SIRT 2, showing significantly increased expression during early and mid secretory phases, while SIRT3, 4, and 7 expressions peaked only during early secretory phase. SIRT 7 expression levels were low throughout the cycle except during the early secretory phase. Sirtuins are functionally diverse, and their

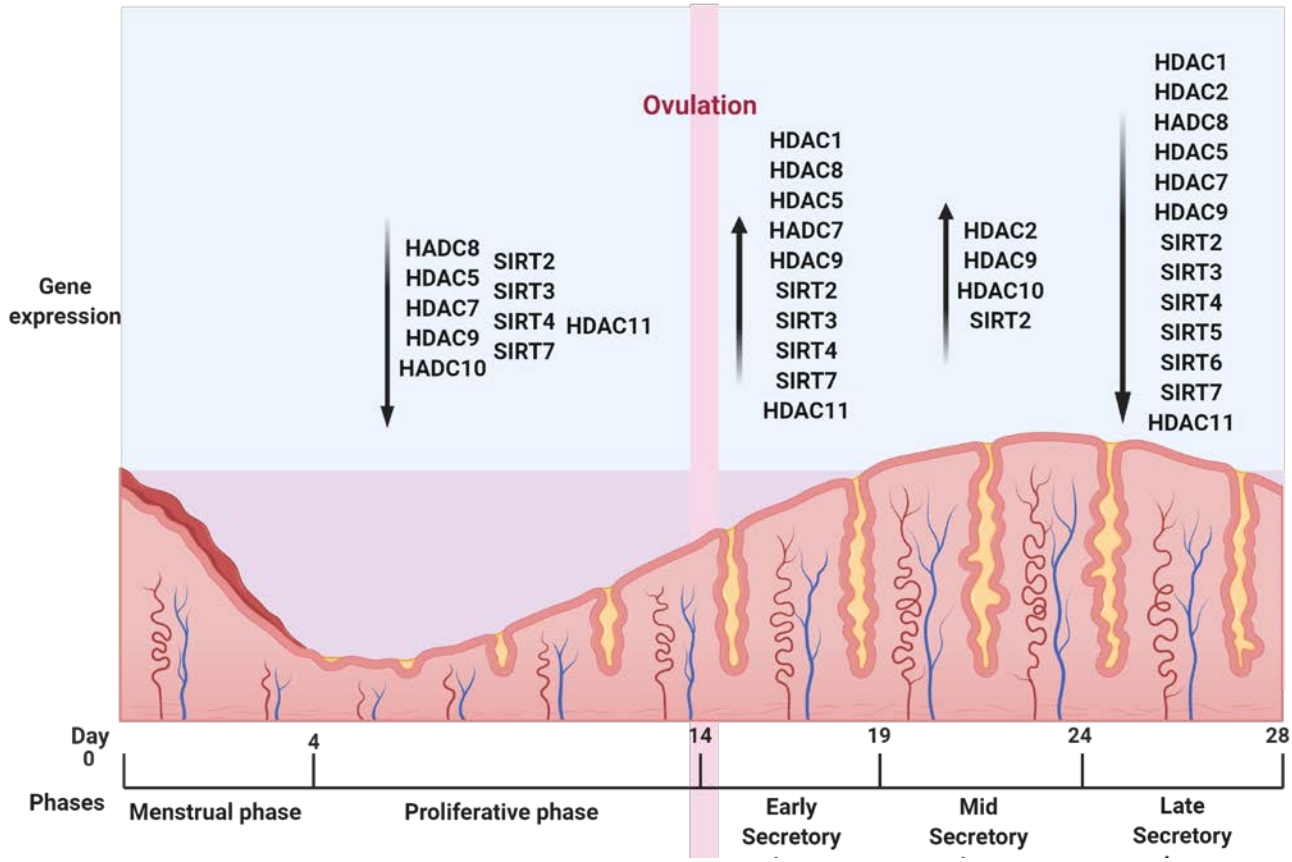

Figure 32: HDAC gene expression throughout the menstrual cycle

expression location determines their functions. To further understand their individual functions in the endometrium, they need to be explored individually in a cell-specific manner (236).

HDAC11 seems to follow a similar trend with significant upregulation in gene expression during the early secretory phase. There are not many studies showing its individual functions of HDAC11 but is most closely related to class 1 HDACs in terms of sequence similarity and functions (167)

4.2 HDAC gene expression changes in response to steroid hormones *in vitro*

I wanted to investigate steroid hormone effects on HDACs in different cell populations in the endometrium. Endometrium widely consists of two types of cells epithelial and stromal cells. AN3 and RL95-2 cells were used instead of the initial decision to use human endometrial epithelial cell line HES as epithelial cell models for this study, due to recent claims of the HES cell line being contaminated (237). RL95-2 cells are often considered a model for receptive epithelium, while AN3 cells are considered models for non-receptive epithelium (238-240). HESC cells were used to study the steroid hormone effects in stromal cells. While there are no previous studies investigating the regulation of HDACs in endometrial epithelial cells, there have been studies looking at class 1 HDACs in endometrial stromal cells(206). Cell specific changes of HDAC expressions in response to hormonal treatment in AN3, RL95-2, and HESCs is important in understanding the roles of these enzymes in different cell types. The cells were treated with estradiol (E_2) for 24hrs and then with E2 and progesterone (P4) for 24hrs, 48hrs, and 72hrs, mimicking the steroidal influence in menstrual cycle phases. The results suggest that there is an effect of steroid hormones on HDAC expression in endometrial epithelial cell line AN3, a peak in gene expression usually observed after 48hrs of treatment with E2+P4 with

a subsequent decrease after that. The HDAC gene expression levels widely differed between the three cell lines.

4.2.1 HDAC expressions in epithelial cells

The responses to treatments were very different in the two epithelial cell lines used. AN3 being a non-receptive epithelial cell model has different cellular functions and requirements compared to a receptive epithelial cell model RL95-2. RL95-2 cells are highly susceptible to steroidal activity due to the presence of high-affinity steroid receptors (230) HDAC mRNA expression in RL95-2 cells widely varied from that of AN3 cell line, suggestive of unique properties and requirements of the receptive epithelium. HDAC1 and 2 expressions were the highest as compared to other HDACs in both RL95-2 and AN3 cells, following suit with the consistent expression in tissue samples. Whereas HDAC3 expression was not observed in RL95-2 cells, there was a significant expression seen in AN3 cell lines. HDAC3 is known to have an essential role in successful implantation to take place; this difference seen between two epithelial cell lines needs to be explored further (211). In AN3 most of the HDACs showed an increase in gene expression at the 48hrs interval of treatment with E2+P4; in RL95-2, some HDACs did show a similar pattern the expression levels were not significant. Most variation was seen in the effect of the HDAC inhibitor TSA. In contrast to RL95-2 the gene expression of HDAC1 was highly upregulated after 24hrs of treatment with TSA+E2 then subsequently reduced after each 24hrs interval in the AN3 cells. HDAC1 expression level increased significantly in RL95-2 cells after 72hrs of treatment with TSA+E2+P4. This increase in HDAC expression levels in TSA and hormone treated groups after 72 hrs was observed in most of the HDACs in RL95-2 which was not evident in AN3. A significant effect of TSA was observed in SIRT2 in AN3 cells. In contrast to RL95-2, SIRT2 expression in AN3 significantly increased after treatment with TSA+E2 for 24hrs in comparison to controls, and only E2 treated cells. A study on the effect of TSA on sirtuins has shown that SIRT2, 4, and 7 are upregulated

in 24hrs treatment with TSA (241), a trend observed in the results for AN3 cells as well, although most significant effects were seen in SIRT2. The above study only reported the effects of TSA till 24hrs of treatment and was performed on neuroblastoma cells. In the present study, a subsequent decrease is seen in levels of SIRT2 and 7 after treatment with TSA+E2+P4. Further studies are needed to determine whether this decrease is a long-term effect of TSA or P4 is affecting the expression levels of SIRTs. It is also interesting to see that RL95-2 does not show this positive effect SIRT2 expression after treatment with TSA in the first 24hrs; there is an increase observed after 72hrs of treatment with TSA+E2+P4. This shows how the HDAC inhibitor induces different responses in different cell types. The effect of TSA is worth exploring in primary cell lines as this varied effect on two different cell types can cause a big difference in its usage in cancer studies. Its seen from the study that steroid hormones do affect the expression of HDACs in the epithelial cells, but the expression can differ with cell lines according to their morphological and functional properties.

4.3 HESC HDAC expression

The HESC cells showed very low HDAC expression levels as compared to AN3 and RL95-2 cells. Endometrial stromal cells are essential for epithelium development and differentiation (242). HDAC1 and 2 were the highest and consistently expressed in HESCs, while HDAC3 had little to no expression, similar to tissue samples. A significant downregulation was observed in HDAC1 after 48hrs of treatment with E2+P4, while there were no significant changes in HDAC2 levels throughout the treatment. The HESCs did not express most of the HDACs, with very high ct values obtained in class 2 HDACs (4, 5, 6, 7, 9, and 10), SIRTs (4, 5, and 7) and HDAC10. Stromal cells have a significant role in decidualization and implantation. A study has shown that inhibition of HDACs using TSA in HESC cells limits trophoblast invasion and causes hindrance in implantation (206). It's interesting to see how the

HDAC are so meagrely expressed in them. However, still, inhibition of HDACs with TSA can cause a drastic effect in the HESCs.

4.4 Limitations

HDACs work in the form of complexes with other HDACs and enzymes, they can act as substrates for multiple enzymes or work on multiple substrates (94, 113). Therefore, it becomes difficult to understand the individual functions of HDACs in regulating the endometrium. The limited tissue sample numbers and lack of time to collect fresh samples was the most significant limitation of the study. The HDAC expression varies between early proliferative to late proliferative and then again in secretory to varied menstruation expression is required, as each sub-cycle stage has varied morphological and functional changes according to the requirement. Tissue mRNA samples and protein samples could be further segregated, but there were limited or no samples for a few cycle stages. Moreover, endometrial tissue includes a mixture of endometrial cells, which are not only composed of epithelial and stromal cells but also uterine leukocytes. A few studies have investigated the effects of HDAC inhibitors on leukocytes and none so far in uterine leukocytes (243, 244). Although there are a few studies investigating the involvement of HDACs in regulating cytokine production in human placenta (227, 245).

This research has separately assessed the effect of steroid hormones on HDAC gene expression in endometrial epithelial and stromal cell models, *in vitro*. The idea was to mimic the hormonal profile during the menstrual cycle. The menstrual cycle events can more ideally be mimicked in endometrial primary explants. A previous study by Dr. Munro did focus on the effect of steroid hormones on histone acetylation in endometrial explants (232), it would also be useful to study the HDAC expression levels in explants in response to steroid hormones. This data combined with *in vitro* cell culture experiments will give us better insight into the effect of hormones on HDACs the human menstrual cycle.

Further studies on primary cell lines would help us better understand the function of HDACs. It should also be noted that the cell lines used as a model for epithelium are cancer cell lines, they can have a different effect as compared healthy epithelial cells.

4.5 Future directions

Studies investigating the localization of the HDACs will help us get further clarity into their functions. Since HDACs are being studied in several endometrial pathologies studying the effect of different HDAC inhibitors would be helpful. Moreover, knockout studies would be very helpful in determining the critical functions of the HDACs. Since different isoforms of proteins were observed in the western blot analysis, further investigation is required to identify the isoforms.

4.6 Conclusion

In this study, significant changes were observed in the human endometrium in HDAC gene and protein expression levels throughout the menstrual cycle. The data imply that HDAC gene expression is usually higher around the ovulation during the early secretory or mid secretory phases, subsequently declining during the late secretory phase. Individual differences in gene expression in different cell populations also show changes in exposure to steroid hormones and exposure time. Different endometrial cells have unique HDAC expression pattern in response to the steroid hormones suggesting, HDACs could have different functions in different cell types. The findings from this research justify further investigations into the role of HDACs in endometrial biology.

5 References

1. Gujral P, Mahajan V, Lissaman AC, Ponnampalam AP. Histone acetylation and the role of histone deacetylases in normal cyclic endometrium. Reproductive Biology and Endocrinology. 2020;18(1):84.

2. Britannica TEoE [Internet]. Encyclopædia Britannica, inc. Uterus; [cited April 16, 2019]. Available from: https://www.britannica.com/science/uterus

3. Eckschlager T. The menstrual cycle. Rennie PIC, editor. London: London : Lloyd-Luke 1976.; 1976.

4. Youssef AF. The uterine isthmus and its sphincter mechanism, a radiographic study: I. The uterine isthmus under normal conditions. American Journal of Obstetrics & Gynecology. 1958;75(6):1305-19.

5. Chaudhry R, Chaudhry K. Anatomy, Abdomen and Pelvis, Uterine Arteries. In: StatPearls. Treasure Island (FL): StatPearls Publishing LLC.; 2020.

6. Chard T, Grudzinskas JG, Grudzinskas J. The uterus. Cambridge University Press; 1994.

7. Cortés J. Jiménez-Ayala M, Jiménez-Ayala Portillo B. Cytopathology of the glandular lesions of the female genital tract. En: Orell SR, editor. Monographs in Clinical Cytology. Vol. 20. Basel (Suiza): Karger Eds.; 2011. ISBN 978-3-8055-9464-6. Progresos de Obstetricia y Ginecología. 2011;54(7).

8. Lessey BA, Young SL. Chapter 9 - Structure, Function, and Evaluation of the Female Reproductive Tract. In: Strauss JF, Barbieri RL, editors. Yen and Jaffe's Reproductive Endocrinology (Eighth Edition). Philadelphia: Content Repository Only!; 2019. p. 206-47.e13.

9. Jiménez-Ayala M, Jiménez-Ayala Portillo B. Cytology of the Normal Endometrium – Cycling and Postmenopausal.

10. Kimball FA. The Endometrium. Dordrecht: Dordrecht : Springer Netherlands 1980.; 1980.

11. Group obotEARW, Griesinger G, Diedrich K, Fauser BCJM, Devroey P. The role of the endometrium and embryo in human implantation. Human Reproduction Update. 2007;13(4):365-77.

12. Curry TE, Jr., Osteen KG. The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. Endocr Rev. 2003;24(4):428-65.

13. Monard M, Marsh C, Schumacher K, Nothnick W. Secretory phase of menstruation and implantation. Frontiers in Women’s Health. 2018;3(4).

14. MacManes MD, Austin SH, Lang AS, Booth A, Farrar V, Calisi RM. Widespread patterns of sexually dimorphic gene expression in an avian hypothalamic-pituitary-gonadal (HPG) axis. Sci Rep. 2017;7:45125.

15. Plachetzki DC, Tsai PS, Kavanaugh SI, Sower SA. Ancient origins of metazoan gonadotropin-releasing hormone and their receptors revealed by phylogenomic analyses. Gen Comp Endocrinol. 2016;234:10-9.

16. Ferin MJG, obstetrics. The hypothalamic-hypophyseal-ovarian axis and the menstrual cycle. 2000;5:1-15.

17. Blair JA, McGee H, Bhatta S, Palm R, Casadesus G. Hypothalamic-pituitary-gonadal axis involvement in learning and memory and Alzheimer's disease: more than "just" estrogen. Front Endocrinol (Lausanne). 2015;6:45.

18. White BA. Endocrine and reproductive physiology. 4th ed.. ed. White BA, Porterfield SP, editors. Philadelphia, PA: Philadelphia, PA : Elsevier/Mosby ©2013.; 2013.

19. Makieva S, Giacomini E, Ottolina J, Sanchez AM, Papaleo E, Vigano P. Inside the Endometrial Cell Signaling Subway: Mind the Gap(s). Int J Mol Sci. 2018;19(9):2477.

20. Gold EB, Bromberger J, Crawford S, Samuels S, Greendale GA, Harlow SD, et al. Factors associated with age at natural menopause in a multiethnic sample of midlife women. Am J Epidemiol. 2001;153(9):865-74.

21. Munro SK, Farquhar CM, Mitchell MD, Ponnampalam AP. Epigenetic regulation of endometrium during the menstrual cycle. Mol Hum Reprod. 2010;16(5):297-310.

22. Khan-Dawood FS. The ovarian cycle. In: Introduction to mammalian reproduction. Springer; 2003. p. 155-86.

23. Balen A. Reproductive Endocrinology. London, UNITED KINGDOM: Royal College of Obstetricians and Gynaecologists; 2007.

24. Mutter GL, Prat J. Pathology of the female reproductive tract. Third edition / edited by George L. Mutter, Jaime Prat.. ed.: Edinburgh : Churchill Livingstone Elsevier. 2014.; 2014.

25. Marsh C, Schumacher K, Nothnick WB, Taylor RN, Monard M. Secretory Phase and Implantation. In: Menstrual Cycle. IntechOpen; 2018.

26. Noyes RW, Hertig AT, Rock J. Dating the Endometrial Biopsy. Fertil Steril. 1950;1(1):3-25.

27. Dallenbach-Hellweg G. The normal histology of the endometrium. In: Histopathology of the Endometrium. Springer; 1971. p. 20-76.

28. Murdock TA, Veras EFT, Kurman RJ, Mazur MT. The Normal Endometrium. In: Murdock TA, Veras EFT, Kurman RJ, Mazur MT, editors. Diagnosis of Endometrial Biopsies and Curettings. Cham: Springer International Publishing; 2019. p. 9-37.

29. Evans J, Salamonsen LA. Inflammation, leukocytes and menstruation. Rev Endocr Metab Disord. 2012;13(4):277-88.

30. Okada H, Tsuzuki T, Murata H. Decidualization of the human endometrium. Reprod Med Biol. 2018;17(3):220-7.

31. Singh M, Chaudhry P, Asselin E. Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors. J Endocrinol. 2011;210(1):5-14.

32. Aplin JD. The cell biological basis of human implantation. Baillieres Best Pract Res Clin Obstet Gynaecol. 2000;14(5):757-64.

33. Maruyama T, Yoshimura Y. Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium. Endocr J. 2008;55(5):795-810.

34. Lois S. My Womban's Life: Understanding Human Endometrial Function. Reproduction. 2018:REP-18-0518.

35. Evans J, Salamonsen LA, Winship A, Menkhorst E, Nie G, Gargett CE, et al. Fertile ground: human endometrial programming and lessons in health and disease. Nat Rev Endocrinol. 2016;12(11):654-67.

36. Schatz F, Guzeloglu-Kayisli O, Arlier S, Kayisli UA, Lockwood CJ. The role of decidual cells in uterine hemostasis, menstruation, inflammation, adverse pregnancy outcomes and abnormal uterine bleeding. Hum Reprod Update. 2016;22(4):497-515.

37. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Seminars in reproductive medicine. 2007;25(6):445-53.

38. Su R-W, Fazleabas AT. Implantation and establishment of pregnancy in human and nonhuman primates. In: Regulation of Implantation and Establishment of Pregnancy in Mammals. Springer; 2015. p. 189-213.

39. Dunn CL, Kelly RW, Critchley HO. Decidualization of the human endometrial stromal cell: an enigmatic transformation. Reprod Biomed Online. 2003;7(2):151-61.

40. Finn CA. Implantation, menstruation and inflammation. Biol Rev Camb Philos Soc. 1986;61(4):313-28.

41. Salamonsen LA, Lathbury LJ. Endometrial leukocytes and menstruation. Hum Reprod Update. 2000;6(1):16-27.

42. Starkey PM, Clover LM, Rees MC. Variation during the menstrual cycle of immune cell populations in human endometrium. Eur J Obstet Gynecol Reprod Biol. 1991;39(3):203-7.

43. Berbic M, Ng CH, Fraser IS. Inflammation and endometrial bleeding. Climacteric. 2014;17 Suppl 2(sup2):47-53.

44. Jabbour HN, Sales KJ, Catalano RD, Norman JE. Inflammatory pathways in female reproductive health and disease. Reproduction. 2009;138(6):903-19.

45. King A, Wellings V, Gardner L, Loke YW. Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. Human Immunology. 1989;24(3):195-205.

46. Salamonsen LA, Lathbury LJ. Endometrial leukocytes and menstruation. Human Reproduction Update. 2000;6(1):16-27.

47. Acar N, Ustunel I, Demir R. Uterine natural killer (uNK) cells and their missions during pregnancy: a review. Acta Histochem. 2011;113(2):82-91.

48. Trundley A, Moffett A. Human uterine leukocytes and pregnancy. Tissue Antigens. 2004;63(1):1-12.

49. Faas MM, de Vos P. Uterine NK cells and macrophages in pregnancy. Placenta. 2017;56:44-52.

50. Saito S, Nishikawa K, Morii T, Enomoto M, Narita N, Motoyoshi K, et al. Cytokine production by CD16–CD56bright natural killer cells in the human early pregnancy decidua. 1993;5(5):559-63.

51. Ferenczy A, Mutter, G. The Endometrial Cycle. Glob libr women's med. 2008.

52. Wilson EW. The menstrual cycle. Rennie PIC, editor. London: London : Lloyd-Luke 1976.; 1976.

53. Salamonsen L. My Womban's Life: Understanding Human Endometrial Function. 2018:REP-18-0518.

54. Thiyagarajan DK JR. Physiology, Menstrual Cycle. StatPearls [Internet].

55. Estrogen. In: Wilson DA, editor. Clinical Veterinary Advisor. Saint Louis: W.B. Saunders; 2012. p. 931.

56. Stillwell W. Chapter 20 - Bioactive Lipids. In: Stillwell W, editor. An Introduction to Biological Membranes (Second Edition). Elsevier; 2016. p. 453-78.

57. Beato M, Klug J. Steroid hormone receptors: an update. Hum Reprod Update. 2000;6(3):225-36.

58. Karl M, Schulte HM, Chrousos GP. Mutation Analysis of Steroid Hormone Receptors. In: de Kloet ER, Sutanto W, editors. Neurobiology of Steroids. Vol. 22, Methods in Neurosciences: Academic Press; 1994. p. 226-41.

59. Hapangama DK, Kamal AM, Bulmer JN. Estrogen receptor beta: the guardian of the endometrium. Hum Reprod Update. 2015;21(2):174-93.

60. Katzenellenbogen BS. Biology and receptor interactions of estriol and estriol derivatives in vitro and in vivo. J Steroid Biochem. 1984;20(4B):1033-7.

61. Greaves E, Collins F, Critchley HO, Saunders PTJHR. ERβ-dependent effects on uterine endothelial cells are cell specific and mediated via Sp1. 2013;28(9):2490-501.

62. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. Mechanisms of estrogen action. Physiol Rev. 2001;81(4):1535-65.

63. Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, et al. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature. 1986;320(6058):134-9.

64. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A. 1996;93(12):5925-30.

65. Van den Veyver IB. Fauser BCJM, Editor-in-Chief; Bouchard P, Hsueh AJW, Rutherford AJW, Simpson JL, Strauss JF 3rd, van Steirteghem A, Section Editors. Reproductive Medicine: Molecular, Cellular and Genetic Fundamentals. Fertil Steril. 2003;80(1):235-6.

66. Critchley HO, Kelly RW, Brenner RM, Baird DTJCe. The endocrinology of menstruation–a role for the immune system. 2001;55(6):701-10.

67. Sato T, Miyagawa S, Iguchi T. Estradiol-17β. In: Handbook of Hormones. Elsevier; 2016. p. 520-e94G-4.

68. Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, et al. Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. Proc Natl Acad Sci U S A. 1997;94(12):6535-40.

69. Matsuzaki S, Fukaya T, Suzuki T, Murakami T, Sasano H, Yajima A. Oestrogen receptor alpha and beta mRNA expression in human endometrium throughout the menstrual cycle. Mol Hum Reprod. 1999;5(6):559-64.

70. Critchley HO, Henderson TA, Kelly RW, Scobie GS, Evans LR, Groome NP, et al. Wild-type estrogen receptor (ERβ1) and the splice variant (ERβcx/β2) are both expressed within the human endometrium throughout the normal menstrual cycle. 2002;87(11):5265-73.

71. Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M, et al. Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. Proc Natl Acad Sci U S A. 2000;97(11):5936-41.

72. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. Cell. 1995;83(6):835-9.

73. Li X, O'Malley BW. Unfolding the action of progesterone receptors. J Biol Chem. 2003;278(41):39261-4.

74. Kastner P, Bocquel MT, Turcotte B, Garnier JM, Horwitz KB, Chambon P, et al. Transient Expression of Human and Chicken Progesterone Receptors Does Not Support Alternative Translational Initiation from a Single Messenger-Rna as the Mechanism Generating 2 Receptor Isoforms. Journal of Biological Chemistry. 1990;265(21):12163-7.

75. Tora L, Gronemeyer H, Turcotte B, Gaub MP, Chambon P. The N-terminal region of the chicken progesterone receptor specifies target gene activation. Nature. 1988;333(6169):185-8.

76. Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM. Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. Science. 2000;289(5485):1751-4.

77. Al-Asmakh MJMEFSJ. Reproductive functions of progesterone. 2007;12(3):147.

78. Tung L, Mohamed MK, Hoeffler JP, Takimoto G, Horwitz KJME. Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors. 1993;7(10):1256-65.

79. Attia GR, Zeitoun K, Edwards D, Johns A, Carr BR, Bulun SE. Progesterone receptor isoform A but not B is expressed in endometriosis. J Clin Endocr Metab. 2000;85(8):2897-902.

80. Mote PA, Balleine RL, McGowan EM, Clarke CL. Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle. J Clin Endocrinol Metab. 1999;84(8):2963-71.

81. Lessey BA. Endometrial receptivity and the window of implantation. Baillieres Best Pract Res Clin Obstet Gynaecol. 2000;14(5):775-88.

82. Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S. Role of nuclear progesterone receptor isoforms in uterine pathophysiology. Hum Reprod Update. 2015;21(2):155-73.

83. Wu C, Morris JR. Genes, genetics, and epigenetics: a correspondence. Science. 2001;293(5532):1103-5.

84. Portela A, Esteller M. Epigenetic modifications and human disease. Nat Biotechnol. 2010;28(10):1057-68.

85. Simmons D. Epigenetic influence and disease. Nature Education. 2008;1(1):6.

86. Urdinguio RG, Sanchez-Mut JV, Esteller M. Epigenetic mechanisms in neurological diseases: genes, syndromes, and therapies. Lancet Neurol. 2009;8(11):1056-72.

87. Kader F, Ghai M, Maharaj L. The effects of DNA methylation on human psychology. Behav Brain Res. 2018;346:47-65.

88. Jin B, Li Y, Robertson KD. DNA methylation: superior or subordinate in the epigenetic hierarchy? Genes Cancer. 2011;2(6):607-17.

89. Kukushkina V, Modhukur V, Suhorutsenko M, Peters M, Magi R, Rahmioglu N, et al. DNA methylation changes in endometrium and correlation with gene expression during the transition from pre-receptive to receptive phase. Sci Rep. 2017;7(1):3916.

90. Gao F, Ma X, Rusie A, Hemingway J, Ostmann AB, Chung D, et al. Epigenetic changes through DNA methylation contribute to uterine stromal cell decidualization. Endocrinology. 2012;153(12):6078-90.

91. Pathak R, Singh P, Ananthakrishnan S, Adamczyk S, Schimmel O, Govind CK. Acetylation-Dependent Recruitment of the FACT Complex and Its Role in Regulating Pol II Occupancy Genome-Wide in Saccharomyces cerevisiae. Genetics. 2018;209(3):743-56.

92. Annunziato AJNE. DNA packaging: nucleosomes and chromatin. 2008;1(1):26.

93. Peterson CL, Laniel MA. Histones and histone modifications. Curr Biol. 2004;14(14):R546-51.

94. Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. Cell Res. 2011;21(3):381-95.

95. Ellenbroek B, Youn J. Environment Challenges and the Brain. In: Ellenbroek B, Youn J, editors. Gene-Environment Interactions in Psychiatry. San Diego: Academic Press; 2016. p. 107-39.

96. Turner BM. Histone acetylation and an epigenetic code. Bioessays. 2000;22(9):836-45.

97. Chrun ES, Modolo F, Daniel FI. Histone modifications: A review about the presence of this epigenetic phenomenon in carcinogenesis. Pathol Res Pract. 2017;213(11):1329-39.

98. Fuks F. DNA methylation and histone modifications: teaming up to silence genes. Curr Opin Genet Dev. 2005;15(5):490-5.

99. Hildmann C, Riester D, Schwienhorst AJAm, biotechnology. Histone deacetylases—an important class of cellular regulators with a variety of functions. 2007;75(3):487-97.

100. Richman R, Chicoine LG, Collini MP, Cook RG, Allis CD. Micronuclei and the cytoplasm of growing Tetrahymena contain a histone acetylase activity which is highly specific for free histone H4. The Journal of cell biology. 1988;106(4):1017-26.

101. Roth SY, Denu JM, Allis CD. Histone acetyltransferases. Annu Rev Biochem. 2001;70(1):81-120.

102. Lee KK, Workman JL. Histone acetyltransferase complexes: one size doesn't fit all. Nat Rev Mol Cell Biol. 2007;8(4):284-95.

103. Sun XJ, Man N, Tan Y, Nimer SD, Wang L. The Role of Histone Acetyltransferases in Normal and Malignant Hematopoiesis. Front Oncol. 2015;5(108):108.

104. Voss AK, Thomas T. MYST family histone acetyltransferases take center stage in stem cells and development. Bioessays. 2009;31(10):1050-61.

105. Bowers EM, Yan G, Mukherjee C, Orry A, Wang L, Holbert MA, et al. Virtual ligand screening of the p300/CBP histone acetyltransferase: identification of a selective small molecule inhibitor. Chem Biol. 2010;17(5):471-82.

106. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou JX, Mizzen CA, et al. Steroid receptor coactivator-1 is a histone acetyltransferase. Nature. 1997;389(6647):194-8.

107. Chen HW, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, et al. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell. 1997;90(3):569-80.

108. Gajer JM, Furdas SD, Grunder A, Gothwal M, Heinicke U, Keller K, et al. Histone acetyltransferase inhibitors block neuroblastoma cell growth in vivo. Oncogenesis. 2015;4(2):e137.

109. Dekker FJ, Haisma HJ. Histone acetyl transferases as emerging drug targets. Drug Discov Today. 2009;14(19-20):942-8.

110. Ait-Si-Ali S, Polesskaya A, Filleur S, Ferreira R, Duquet A, Robin P, et al. CBP/p300 histone acetyl-transferase activity is important for the G1/S transition. Oncogene. 2000;19(20):2430-7.

111. Yan G, Eller MS, Elm C, Larocca CA, Ryu B, Panova IP, et al. Selective inhibition of p300 HAT blocks cell cycle progression, induces cellular senescence, and inhibits the DNA damage response in melanoma cells. J Invest Dermatol. 2013;133(10):2444-52.

112. Hammett R, Shoemake CJMC. In Silico Design and Optimisation of Phytoalexin Resveratrol Polyphenolic Analogs as Inhibitors of Histone Acetyltransferase for the Management of Tumor Growth. 2018;8:242-9.

113. Seto E, Yoshida M. Erasers of histone acetylation: the histone deacetylase enzymes. Cold Spring Harb Perspect Biol. 2014;6(4):a018713.

114. Hadley M, Noonepalle S, Banik D, Villagra A. Functional Analysis of HDACs in Tumorigenesis. In: Brosh JRM, editor. Protein Acetylation: Methods and Protocols. New York, NY: Springer New York; 2019. p. 279-307.

115. Lee J, R SH. Cancer Epigenetics: Mechanisms and Crosstalk of a HDAC Inhibitor, Vorinostat. Chemotherapy (Los Angel). 2013;2(111):14934.

116. Taunton J, Hassig CA, Schreiber SL. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science. 1996;272(5260):408-11.

117. Hildmann C, Wegener D, Riester D, Hempel R, Schober A, Merana J, et al. Substrate and inhibitor specificity of class 1 and class 2 histone deacetylases. J Biotechnol. 2006;124(1):258-70.

118. Waltregny D, De Leval L, Glenisson W, Ly Tran S, North BJ, Bellahcene A, et al. Expression of histone deacetylase 8, a class I histone deacetylase, is restricted to cells showing smooth muscle differentiation in normal human tissues. Am J Pathol. 2004;165(2):553-64.

119. Reichert N, Choukrallah MA, Matthias P. Multiple roles of class I HDACs in proliferation, differentiation, and development. Cell Mol Life Sci. 2012;69(13):2173-87.

120. Liu XF, Bagchi MK. Recruitment of distinct chromatin-modifying complexes by tamoxifen-complexed estrogen receptor at natural target gene promoters in vivo. J Biol Chem. 2004;279(15):15050-8.

121. Brunmeir R, Lagger S, Seiser C. Histone deacetylase 1 and 2-controlled embryonic development and cell differentiation. Int J Dev Biol. 2009;53(2-3):275-89.

122. Taplick J, Kurtev V, Kroboth K, Posch M, Lechner T, Seiser C. Homo-oligomerisation and nuclear localisation of mouse histone deacetylase 1. J Mol Biol. 2001;308(1):27-38.

123. Luo Y, Jian W, Stavreva D, Fu XQ, Hager G, Bungert J, et al. Trans-regulation of Histone Deacetylase Activities through Acetylation. Journal of Biological Chemistry. 2009;284(50):34901-10.

124. Morris MJ, Monteggia LM. Unique functional roles for class I and class II histone deacetylases in central nervous system development and function. Int J Dev Neurosci. 2013;31(6):370-81.

125. Lagger G, O'Carroll D, Rembold M, Khier H, Tischler J, Weitzer G, et al. Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J. 2002;21(11):2672-81.

126. Montgomery RL, Davis CA, Potthoff MJ, Haberland M, Fielitz J, Qi X, et al. Histone deacetylases 1 and 2 redundantly regulate cardiac morphogenesis, growth, and contractility. Genes Dev. 2007;21(14):1790-802.

127. Weichert W, Denkert C, Noske A, Darb-Esfahani S, Dietel M, Kalloger SE, et al. Expression of class I histone deacetylases indicates poor prognosis in endometrioid subtypes of ovarian and endometrial carcinomas. Neoplasia. 2008;10(9):1021-7.

128. Krusche CA, Vloet AJ, Classen-Linke I, von Rango U, Beier HM, Alfer J. Class I histone deacetylase expression in the human cyclic endometrium and endometrial adenocarcinomas. Hum Reprod. 2007;22(11):2956-66.

129. Fischle W, Dequiedt F, Fillion M, Hendzel MJ, Voelter W, Verdin EJJoBC. Human HDAC7 Histone Deacetylase Activity Is Associated with HDAC3in Vivo. 2001;276(38):35826-35.

130. Grozinger CM, Hassig CA, Schreiber SL. Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proc Natl Acad Sci U S A. 1999;96(9):4868-73.

131. Mathias RA, Guise AJ, Cristea IM. Post-translational modifications regulate class IIa histone deacetylase (HDAC) function in health and disease. Mol Cell Proteomics. 2015;14(3):456-70.

132. Zhou B, Margariti A, Xu Q. The Role of Epigenetics in Cardiovascular Disease. In: Tollefsbol TO, editor. Epigenetics in Human Disease. San Diego: Academic Press; 2012. p. 395-414.

133. Parra M. Class IIa HDACs - new insights into their functions in physiology and pathology. The FEBS journal. 2015;282(9):1736-44.

134. Di Giorgio E, Dalla E, Franforte E, Paluvai H, Minisini M, Trevisanut M, et al. Different class IIa HDACs repressive complexes regulate specific epigenetic responses related to cell survival in leiomyosarcoma cells. Nucleic Acids Res. 2020;48(2):646-64.

135. Fitzsimons HL. The Class IIa histone deacetylase HDAC4 and neuronal function: Nuclear nuisance and cytoplasmic stalwart? Neurobiol Learn Mem. 2015;123:149-58.

136. Di Giorgio E, Brancolini C. Regulation of class IIa HDAC activities: it is not only matter of subcellular localization. Epigenomics. 2016;8(2):251-69.

137. Wright LH, Menick DR. A class of their own: exploring the nondeacetylase roles of class IIa HDACs in cardiovascular disease. Am J Physiol Heart Circ Physiol. 2016;311(1):H199-206.

138. Kaluza D, Kroll J, Gesierich S, Yao TP, Boon RA, Hergenreider E, et al. Class IIb HDAC6 regulates endothelial cell migration and angiogenesis by deacetylation of cortactin. EMBO J. 2011;30(20):4142-56.

139. Ahn MY, Chung HY, Choi WS, Lee BM, Yoon S, Kim HS. Anti-tumor effect of apicidin on Ishikawa human endometrial cancer cells both in vitro and in vivo by blocking histone deacetylase 3 and 4. Int J Oncol. 2010;36(1):125-31.

140. Yang XJ, Gregoire S. Class II histone deacetylases: from sequence to function, regulation, and clinical implication. Mol Cell Biol. 2005;25(8):2873-84.

141. Brindisi M, Saraswati AP, Brogi S, Gemma S, Butini S, Campiani GJJomc. Old but Gold: Tracking the New Guise of Histone Deacetylase 6 (HDAC6) Enzyme as a Biomarker and Therapeutic Target in Rare Diseases. 2019.

142. Simões-Pires C, Zwick V, Nurisso A, Schenker E, Carrupt P-A, Cuendet MJMn. HDAC6 as a target for neurodegenerative diseases: what makes it different from the other HDACs? 2013;8(1):7.

143. Seidel C, Schnekenburger M, Dicato M, Diederich M. Histone deacetylase 6 in health and disease. Epigenomics. 2015;7(1):103-18.

144. Zhang Y, Li N, Caron C, Matthias G, Hess D, Khochbin S, et al. HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. EMBO J. 2003;22(5):1168-79.

145. Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, et al. HDAC6 is a microtubule-associated deacetylase. Nature. 2002;417(6887):455-8.

146. Haggarty SJ, Koeller KM, Wong JC, Grozinger CM, Schreiber SL. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci U S A. 2003;100(8):4389-94.

147. Haiching M, Wang Y. Selective Inhibition of HDAC6 and HDAC8, and Methods of Use Thereof. Google Patents; 2017.

148. Rossaert E, Van Den Bosch L. HDAC6 inhibitors: Translating genetic and molecular insights into a therapy for axonal CMT. Brain research. 2020;1733:146692.

149. Hai Y, Shinsky SA, Porter NJ, Christianson DW. Histone deacetylase 10 structure and molecular function as a polyamine deacetylase. Nat Commun. 2017;8:15368.

150. Kao HY, Lee CH, Komarov A, Han CC, Evans RM. Isolation and characterization of mammalian HDAC10, a novel histone deacetylase. J Biol Chem. 2002;277(1):187-93.

151. Obayashi H, Nagano Y, Takahashi T, Seki T, Tanaka S, Sakai N, et al. Histone deacetylase 10 knockout activates chaperone-mediated autophagy and accelerates the decomposition of its substrate. Biochem Biophys Res Commun. 2020;523(1):246-52.

152. Oehme I, Linke JP, Bock BC, Milde T, Lodrini M, Hartenstein B, et al. Histone deacetylase 10 promotes autophagy-mediated cell survival. Proc Natl Acad Sci U S A. 2013;110(28):E2592-601.

153. Radhakrishnan R, Li YX, Xiang SY, Yuan FH, Yuan ZG, Telles E, et al. Histone Deacetylase 10 Regulates DNA Mismatch Repair and May Involve the Deacetylation of MutS Homolog 2. Journal of Biological Chemistry. 2015;290(37):22795-804.

154. Yang Y, Huang Y, Wang Z, Wang HT, Duan B, Ye D, et al. HDAC10 promotes lung cancer proliferation via AKT phosphorylation. Oncotarget. 2016;7(37):59388-401.

155. Islam MM, Banerjee T, Packard CZ, Kotian S, Selvendiran K, Cohn DE, et al. HDAC10 as a potential therapeutic target in ovarian cancer. Gynecologic oncology. 2017;144(3):613-20.

156. Dai Y, Faller DV. Transcription Regulation by Class III Histone Deacetylases (HDACs)-Sirtuins. Transl Oncogenomics. 2008;3:53-65.

157. Edatt L, Poyyakkara A, Raji GR, Ramachandran V, Shankar SS, Kumar VBS. Role of Sirtuins in Tumor Angiogenesis. Front Oncol. 2019;9:1516.

158. Chang HC, Guarente L. SIRT1 and other sirtuins in metabolism. Trends Endocrinol Metab. 2014;25(3):138-45.

159. Guarente L. Calorie restriction and sirtuins revisited. Genes Dev. 2013;27(19):2072-85.

160. Perrini S, Porro S, Nigro P, Cignarelli A, Caccioppoli C, Genchi VA, et al. Reduced SIRT1 and SIRT2 expression promotes adipogenesis of human visceral adipose stem cells and associates with accumulation of visceral fat in human obesity. Int J Obes (Lond). 2020;44(2):307-19.

161. Quan Y, Park W, Jin J, Kim W, Park SK, Kang KPJIJoMS. Sirtuin 3 Activation by Honokiol Decreases Unilateral Ureteral Obstruction-Induced Renal Inflammation and Fibrosis via Regulation of Mitochondrial Dynamics and the Renal NF-κBTGF-β1/Smad Signaling Pathway. 2020;21(2):402.

162. Liu Y, Zhang Y, Zhu K, Chi S, Wang C, Xie A. Emerging Role of Sirtuin 2 in Parkinson's Disease. Frontiers in aging neuroscience. 2019;11:372.

163. Min Z, Gao J, Yu Y. The Roles of Mitochondrial SIRT4 in Cellular Metabolism. Front Endocrinol (Lausanne). 2018;9(783):783.

164. Kumar S, Lombard DB. Functions of the sirtuin deacylase SIRT5 in normal physiology and pathobiology. Crit Rev Biochem Mol Biol. 2018;53(3):311-34.

165. Onn L, Portillo M, Ilic S, Cleitman G, Stein D, Kaluski S, et al. SIRT6 is a DNA double-strand break sensor. Elife. 2020;9:e51636.

166. Wu D, Li Y, Zhu KS, Wang H, Zhu WG. Advances in Cellular Characterization of the Sirtuin Isoform, SIRT7. Front Endocrinol (Lausanne). 2018;9:652.

167. Aldana-Masangkay GI, Sakamoto KM. The role of HDAC6 in cancer. J Biomed Biotechnol. 2011;2011:875824.

168. Sahakian E, Woan K, Villagra A, Sotomayor EM. Chapter 22 - Epigenetic Approaches: Emerging Role of Histone Deacetylase Inhibitors in Cancer Immunotherapy. In: Prendergast GC, Jaffee EM, editors. Cancer Immunotherapy (Second Edition). San Diego: Academic Press; 2013. p. 353-72.

169. Sahakian E, Woan K, Villagra A, Sotomayor EM. Epigenetic Approaches. In: Prendergast GC, Jaffee EM, editors. Cancer Immunotherapy. San Diego: Academic Press; 2013. p. 353-72.

170. Cao J, Sun L, Aramsangtienchai P, Spiegelman NA, Zhang X, Huang W, et al. HDAC11 regulates type I interferon signaling through defatty-acylation of SHMT2. Proc Natl Acad Sci U S A. 2019;116(12):5487-92.

171. Kim HJ, Bae SC. Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. Am J Transl Res. 2011;3(2):166-79.

172. Eckschlager T, Plch J, Stiborova M, Hrabeta J. Histone Deacetylase Inhibitors as Anticancer Drugs. Int J Mol Sci. 2017;18(7):1414.

173. Suraweera A, O'Byrne KJ, Richard DJ. Combination Therapy With Histone Deacetylase Inhibitors (HDACi) for the Treatment of Cancer: Achieving the Full Therapeutic Potential of HDACi. Front Oncol. 2018;8:92.

174. Gargett CE, Nguyen HP, Ye L. Endometrial regeneration and endometrial stem/progenitor cells. Rev Endocr Metab Disord. 2012;13(4):235-51.

175. Gargett CE, Schwab KE, Deane JA. Endometrial stem/progenitor cells: the first 10 years. Hum Reprod Update. 2016;22(2):137-63.

176. Yakushiji N, Yokoyama H, Tamura K. Repatterning in amphibian limb regeneration: A model for study of genetic and epigenetic control of organ regeneration. Semin Cell Dev Biol. 2009;20(5):565-74.

177. Chung Y-L. Histone hyperacetylating agents for promoting wound healing and preventing scar formation. Google Patents; 2015.

178. Figueira PG, Abrao MS, Krikun G, Taylor HS. Stem cells in endometrium and their role in the pathogenesis of endometriosis. Ann N Y Acad Sci. 2011;1221(1):10-7.

179. Reyes M, Verfaillie CM. Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. Ann N Y Acad Sci. 2001;938:231-3; discussion 3-5.

180. Wolf M, Kiesel L, Götte M. Stammzellen im Endometrium. Gynäkologische Endokrinologie. 2009;7(3):185-9.

181. Gargett CE, Ye L. Endometrial reconstruction from stem cells. Fertil Steril. 2012;98(1):11-20.

182. Li X, Zhao X. Epigenetic regulation of mammalian stem cells. Stem Cells Dev. 2008;17(6):1043-52.

183. Huang B, Li G, Jiang XH. Fate determination in mesenchymal stem cells: a perspective from histone-modifying enzymes. Stem Cell Res Ther. 2015;6(1):35.

184. Xu L, Xing Q, Huang T, Zhou J, Liu T, Cui Y, et al. HDAC1 Silence Promotes Neuroprotective Effects of Human Umbilical Cord-Derived Mesenchymal Stem Cells in a Mouse Model of Traumatic Brain Injury via PI3K/AKT Pathway. Front Cell Neurosci. 2018;12:498.

185. Park SY, Phorl S, Jung S, Sovannarith K, Lee SI, Noh S, et al. HDAC6 deficiency induces apoptosis in mesenchymal stem cells through p53 K120 acetylation. Biochem Biophys Res Commun. 2017;494(1-2):51-6.

186. Tan J, Lu J, Huang W, Dong Z, Kong C, Li L, et al. Genome-wide analysis of histone H3 lysine9 modifications in human mesenchymal stem cell osteogenic differentiation. PLoS One. 2009;4(8):e6792.

187. Verdi J, Tan A, Shoae-Hassani A, Seifalian AM. Endometrial stem cells in regenerative medicine. J Biol Eng. 2014;8(1):20.

188. Ding D-C, Shyu W-C, Lin S-ZJCt. Mesenchymal stem cells. 2011;20(1):5-14.

189. Lu J, Kong X, Luo C, Li KK. Application of epigenome-modifying small molecules in induced pluripotent stem cells. Med Res Rev. 2013;33(4):790-822.

190. Mashayekhi P, Noruzinia M, Zeinali S, Khodaverdi S. Endometriotic Mesenchymal Stem Cells Epigenetic Pathogenesis: Deregulation of miR-200b, miR-145, and let7b in A Functional Imbalanced Epigenetic Disease. Cell journal. 2019;21(2):179-85.

191. Taghizadeh M, Noruzinia M. Lovastatin Reduces Stemness via Epigenetic Reprograming of BMP2 and GATA2 in Human Endometrium and Endometriosis. Cell journal. 2017;19(1):50-64.

192. Shukla V, Vaissiere T, Herceg Z. Histone acetylation and chromatin signature in stem cell identity and cancer. Mutat Res. 2008;637(1-2):1-15.

193. Gargett CE, Rogers PA. Human endometrial angiogenesis. Reproduction. 2001;121(2):181-6.

194. Hayashi A, Horiuchi A, Kikuchi N, Hayashi T, Fuseya C, Suzuki A, et al. Type-specific roles of histone deacetylase (HDAC) overexpression in ovarian carcinoma: HDAC1 enhances cell proliferation and HDAC3 stimulates cell migration with downregulation of E-cadherin. Int J Cancer. 2010;127(6):1332-46.

195. Samartzis EP, Noske A, Samartzis N, Fink D, Imesch P. The expression of histone deacetylase 1, but not other class I histone deacetylases, is significantly increased in endometriosis. Reprod Sci. 2013;20(12):1416-22.

196. Ren J, Zhang J, Cai H, Li Y, Zhang YL, Zhang XZ, et al. HDAC as a Therapeutic Target for Treatment of Endometrial Cancers. Curr Pharm Design. 2014;20(11):1847-56.

197. Hrzenjak A, Moinfar F, Kremser ML, Strohmeier B, Staber PB, Zatloukal K, et al. Valproate inhibition of histone deacetylase 2 affects differentiation and decreases proliferation of endometrial stromal sarcoma cells. Mol Cancer Ther. 2006;5(9):2203-10.

198. Fakhry H, Miyamoto T, Kashima H, Suzuki A, Ke H, Konishi I, et al. Immunohistochemical detection of histone deacetylases in endometrial carcinoma: involvement of histone deacetylase 2 in the proliferation of endometrial carcinoma cells. Hum Pathol. 2010;41(6):848-58.

199. Imesch P, Fink D, Fedier A. Romidepsin reduces histone deacetylase activity, induces acetylation of histones, inhibits proliferation, and activates apoptosis in immortalized epithelial endometriotic cells. Fertil Steril. 2010;94(7):2838-42.

200. Yi TZ, Li J, Han X, Guo J, Qu Q, Guo L, et al. DNMT inhibitors and HDAC inhibitors regulate E-cadherin and Bcl-2 expression in endometrial carcinoma in vitro and in vivo. Chemotherapy. 2012;58(1):19-29.

201. Ropero S, Esteller M. The role of histone deacetylases (HDACs) in human cancer. Mol Oncol. 2007;1(1):19-25.

202. Jin G, Bausch D, Knightly T, Liu Z, Li Y, Liu B, et al. Histone deacetylase inhibitors enhance endothelial cell sprouting angiogenesis in vitro. Surgery. 2011;150(3):429-35.

203. Chen Y, Chang Y, Yao S. Role of angiogenesis in endometrial repair of patients with severe intrauterine adhesion. Int J Clin Exp Pathol. 2013;6(7):1343-50.

204. Tamura I, Ohkawa Y, Sato T, Suyama M, Jozaki K, Okada M, et al. Genome-wide analysis of histone modifications in human endometrial stromal cells. Mol Endocrinol. 2014;28(10):1656-69.

205. van den Brûle F, Berndt S, Simon N, Coulon C, Le Goarant J, Munaut C, et al. Trophoblast invasion and placentation: molecular mechanisms and regulation. In: Immunology of Gametes and Embryo Implantation. Vol. 88: Karger Publishers; 2005. p. 163-80.

206. Estella C, Herrer I, Atkinson SP, Quinonero A, Martinez S, Pellicer A, et al. Inhibition of histone deacetylase activity in human endometrial stromal cells promotes extracellular matrix remodelling and limits embryo invasion. PLoS One. 2012;7(1):e30508.

207. Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol. 2014;15(12):786-801.

208. Lala PK, Chakraborty C. Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury. Placenta. 2003;24(6):575-87.

209. Knofler M. Critical growth factors and signalling pathways controlling human trophoblast invasion. Int J Dev Biol. 2010;54(2-3):269-80.

210. Sakai N, Maruyama T, Sakurai R, Masuda H, Yamamoto Y, Shimizu A, et al. Involvement of histone acetylation in ovarian steroid-induced decidualization of human endometrial stromal cells. J Biol Chem. 2003;278(19):16675-82.

211. Kim TH, Yoo JY, Choi KC, Shin JH, Leach RE, Fazleabas AT, et al. Loss of HDAC3 results in nonreceptive endometrium and female infertility. Sci Transl Med. 2019;11(474):eaaf7533.

212. Clocchiatti A, Florean C, Brancolini C. Class IIa HDACs: from important roles in differentiation to possible implications in tumourigenesis. J Cell Mol Med. 2011;15(9):1833-46.

213. Weichert W. HDAC expression and clinical prognosis in human malignancies. Cancer Lett. 2009;280(2):168-76.

214. Ahn MY, Lee J, Na YJ, Choi WS, Lee BM, Kang KW, et al. Mechanism of apicidin-induced cell cycle arrest and apoptosis in Ishikawa human endometrial cancer cells. Chem Biol Interact. 2009;179(2-3):169-77.

215. Li LH, Zhang PR, Cai PY, Li ZC. Histone deacetylase inhibitor, Romidepsin (FK228) inhibits endometrial cancer cell growth through augmentation of p53-p21 pathway. Biomed Pharmacother. 2016;82:161-6.

216. Bergada L, Sorolla A, Yeramian A, Eritja N, Mirantes C, Matias-Guiu X, et al. Combination of Vorinostat and caspase-8 inhibition exhibits high anti-tumoral activity on endometrial cancer cells. Mol Oncol. 2013;7(4):763-75.

217. Yang S, Jia Y, Liu X, Winters C, Wang X, Zhang Y, et al. Systematic dissection of the mechanisms underlying progesterone receptor downregulation in endometrial cancer. Oncotarget. 2014;5(20):9783-97.

218. Yang S, Xiao X, Jia Y, Liu X, Zhang Y, Wang X, et al. Epigenetic modification restores functional PR expression in endometrial cancer cells. Curr Pharm Des. 2014;20(11):1874-80.

219. Fukuda T, Wada-Hiraike O, Oda K, Tanikawa M, Makii C, Inaba K, et al. Putative tumor suppression function of SIRT6 in endometrial cancer. FEBS Lett. 2015;589(17):2274-81.

220. Bartosch C, Monteiro-Reis S, Almeida-Rios D, Vieira R, Castro A, Moutinho M, et al. Assessing sirtuin expression in endometrial carcinoma and non-neoplastic endometrium. Oncotarget. 2016;7(2):1144-54.

221. Kawano Y, Nasu K, Li H, Tsuno A, Abe W, Takai N, et al. Application of the histone deacetylase inhibitors for the treatment of endometriosis: histone modifications as pathogenesis and novel therapeutic target. Hum Reprod. 2011;26(9):2486-98.

222. Colon-Diaz M, Baez-Vega P, Garcia M, Ruiz A, Monteiro JB, Fourquet J, et al. HDAC1 and HDAC2 are differentially expressed in endometriosis. Reprod Sci. 2012;19(5):483-92.

223. Xiaomeng X, Ming Z, Jiezhi M, Xiaoling F. Aberrant histone acetylation and methylation levels in woman with endometriosis. Archives of gynecology and obstetrics. 2013;287(3):487-94.

224. Taguchi A, Wada-Hiraike O, Kawana K, Koga K, Yamashita A, Shirane A, et al. Resveratrol suppresses inflammatory responses in endometrial stromal cells derived from endometriosis: a possible role of the sirtuin 1 pathway. J Obstet Gynaecol Res. 2014;40(3):770-8.

225. Kim TH, Yoo J-Y, Choi K-C, Shin J-H, Leach RE, Fazleabas AT, et al. Loss of HDAC3 results in nonreceptive endometrium and female infertility. Science Translational Medicine. 2019;11(474):eaaf7533.

226. Ren J, Zhang J, Cai H, Li Y, Zhang Y, Zhang X, et al. HDAC as a therapeutic target for treatment of endometrial cancers. Curr Pharm Des. 2014;20(11):1847-56.

227. Munro SK. The role of histone deacetylases in cytokine regulation in human gestational tissues. 2013.

228. Krikun G, Mor G, Alvero A, Guller S, Schatz F, Sapi E, et al. A novel immortalized human endometrial stromal cell line with normal progestational response. Endocrinology. 2004;145(5):2291-6.

229. Dawe CJ, Banfield WG, Morgan WD, Slatick MS, Curth HO. Growth in Continuous Culture, and in Hamsters, of Cells from a Neoplasm Associated with Acanthosis Nigricans. Journal of the National Cancer Institute. 1964;33:441-56.

230. Way DL, Grosso DS, Davis JR, Surwit EA, Christian CD. Characterization of a new human endometrial carcinoma (RL95-2) established in tissue culture. In Vitro. 1983;19(3 Pt 1):147-58.

231. Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res. 2003;31(24):e154.

232. Munro SK. Histone acetylation in the human endometrium varies with stage of the mentrual cycle 2009.

233. Yamaguchi T, Cubizolles F, Zhang Y, Reichert N, Kohler H, Seiser C, et al. Histone deacetylases 1 and 2 act in concert to promote the G1-to-S progression. Genes Dev. 2010;24(5):455-69.

234. Xie D, Zhu J, Liu Q, Li J, Song M, Wang K, et al. Dysregulation of HDAC9 Represses Trophoblast Cell Migration and Invasion Through TIMP3 Activation in Preeclampsia. Am J Hypertens. 2019;32(5):515-23.

235. Song CL, Zhu SC, Wu CY, Kang JH. Histone Deacetylase (HDAC) 10 Suppresses Cervical Cancer Metastasis through Inhibition of Matrix Metalloproteinase (MMP) 2 and 9 Expression. Journal of Biological Chemistry. 2013;288(39):28021-33.

236. Bartosch C, Monteiro-Reis S, Almeida-Rios D, Vieira R, Castro A, Moutinho M, et al. Assessing sirtuin expression in endometrial carcinoma and non-neoplastic endometrium. Oncotarget; Vol 7, No 2. 2015.

237. Kniss DA, Summerfield TL. Discovery of HeLa Cell Contamination in HES Cells: Call for Cell Line Authentication in Reproductive Biology Research. Reprod Sci. 2014;21(8):1015-9.

238. Thie M, Herter P, Pommerenke H, Durr F, Sieckmann F, Nebe B, et al. Adhesiveness of the free surface of a human endometrial monolayer for trophoblast as related to actin cytoskeleton. Molecular Human Reproduction. 1997;3(4):275-83.

239. Thie M, Denker HW. In vitro studies on endometrial adhesiveness for trophoblast: cellular dynamics in uterine epithelial cells. Cells Tissues Organs. 2002;172(3):237-52.

240. Thie M, Rospel R, Dettmann W, Benoit M, Ludwig M, Gaub HE, et al. Interactions between trophoblast and uterine epithelium: monitoring of adhesive forces. Human Reproduction. 1998;13(11):3211-9.

241. Kyrylenko S, Kyrylenko O, Suuronen T, Salminen A. Differential regulation of the Sir2 histone deacetylase gene family by inhibitors of class I and II histone deacetylases. Cell Mol Life Sci. 2003;60(9):1990-7.

242. Cooke PS, Uchima FD, Fujii DK, Bern HA, Cunha GR. Restoration of normal morphology and estrogen responsiveness in cultured vaginal and uterine epithelia transplanted with stroma. Proc Natl Acad Sci U S A. 1986;83(7):2109-13.

243. Sweet MJ, Shakespear MR, Kamal NA, Fairlie DPJI, biology c. HDAC inhibitors: modulating leukocyte differentiation, survival, proliferation and inflammation. 2012;90(1):14-22.

244. Zheng H, Zhao W, Yan C, Watson CC, Massengill M, Xie M, et al. HDAC inhibitors enhance T-cell chemokine expression and augment response to PD-1 immunotherapy in lung adenocarcinoma. 2016;22(16):4119-32.

245. Mitchell MD, Ponnampalam AP, Rice GEJMoi. Epigenetic regulation of cytokine production in human amnion and villous placenta. 2012;2012.