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Targeting Epigenetic Mechanisms in Endometriosis

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TARGETING EPIGENETIC MECHANISMS IN ENDOMETRIOSIS

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We, the faculty supervising the work of Sarah Elizabeth Brunty, affirm that the dissertation, *Targeting epigenetic mechanisms in endometriosis,* meets the high academic standards for original scholarship and creative work established by the Biomedical Research Graduate Program and the Joan C. Edwards School of Medicine. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

I dedicate this thesis to my mom, Sherry Binion. While life took you too early and you are not able to see me reach the finish line, I know you have been there every step of the way. On the days when I didn't feel like I was doing anything right, I thought of you and how at the end of all of this I could finally tell you that I am now a doctor. I know how proud you were of me for going for this and how proud you would be in all the things that I have done since.

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ABSTRACT

Endometriosis is a complex and elusive gynecological disease in which the inner lining of the uterus grows in locations outside of the uterus and forms lesions. It is known to affect 1 in 9 women of reproductive age worldwide. Symptoms of endometriosis include severe pain, heavy periods, and infertility. While multiple theories of origin exist, none fully encompass all aspects of the disease, although all theories agree that this is an inflammation-driven disease. Due to this, many researchers are turning towards epigenetics to explain the initiation and progression of endometriosis. However, what is causing these epigenetic changes is still a mystery. We hypothesize that peritoneal fluid (PF) from women with endometriosis, full of chemokines, cytokines, and inflammatory molecules, is the initiator behind the changes seen, specifically the Polycomb repressive complex 2 (PRC2) and its catalytic subunit Enhancer of zeste homolog 2 (EZH2). We noted that there was an upregulation of the PRC2 complex and EZH2 in tissue from women with endometriosis. This upregulation was also seen when endometrial cells were treated with PF from women with endometriosis. miR-155 was also shown to be upregulated in the endometrial lesions and endo PF treated cells examined. Interactions between EZH2 and other genes that may be mechanistically working in the pathogenesis of endometriosis were seen through the use of ChIP-qPCR. The results of this study showed us that the PF from women with endometriosis is playing a role in initiating epigenetic mechanisms as well as providing us a potential novel cross-talk between miR-155 and the PRC2 complex. We used the results of upregulation of EZH2 due to PF from women with endometriosis and examined whether these changes could be inhibited or reversed through targeting an inflammatory pathway, the CXCR4-CXCL12-CXCR7 axis, in endometriosis through the use of a CXCR4 agonist AMD3100. We also examined if the EZH2 inhibitor GSK126 would target this inflammatory pathway. Both

cells and tissues from women without endometriosis were treated with a combination of control or endo PF and/or AMD3100 or GSK126 in order to examine gene expression as well as changes in cell proliferation and migration. Endometrial PF was shown to increase the expression of EZH2, as well as the proliferation of the cells. When AMD3100 or GSK126 was added to the cells and the tissues in the presence of endometriosis PF, dual results were noted, suggesting that a one-sided approach, targeting just the inflammation or just the epigenetic mechanisms is not the correct approach for treatment. The best alteration of gene expression, suppression of cell proliferation, and suppression of migration was noted when combining the two compounds for a dual-sided treatment. Overall, the studies presented in this thesis show that both epigenetic mechanisms and inflammatory pathways drive the progression of endometriosis. Further studies will examine the effects that the compounds used in these studies have in animal models of endometriosis where lesion growth and pain levels will be observed.

CHAPTER 1

INFLAMMATION AND EPIGENETICS IN ENDOMETRIOSIS

Some information from this chapter has been published in a review in *Annals of Translational Medicine*.
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ABSTRACT

Endometriosis is a complex and elusive disease that affects over 200 million women of childbearing age worldwide. One of the main symptoms of this disease is the pain that the affected women experience. While there are theories regarding the origin of endometriosis, none fully encompass all of the aspects that are clinically observed. The current understanding is that endometriosis is a hormone-sensitive inflammatory disorder. Genetic associations for endometriosis have been suggested but are unproven. Due to these gaps in knowledge of its etiology, recent investigations have explored whether epigenetic mechanisms may be at play in the initiation and progression of this disease. The focus of this project was to study the epigenetic regulation of key inflammatory pathways such as FOXP3 and CXCL12-CXCR4 axis in endometriosis. This chapter provides an overview of what endometriosis is, the current etiological theories, and the role of steroid hormones, inflammation, and oxidative stress in its etiology. Finally, a summary of how epigenetics may be involved in this disease will be discussed. A better understanding of the mechanisms involved in the pathophysiology of this disease will help improve early diagnosis as well as treatment.

INTRODUCTION

Endometriosis is a gynecological disease that is known to affect 1 in 9 women of childbearing age, however, this number could be higher due to the fact that 20-25% of women who have endometriosis show no signs or symptoms [1,2], and hence there is a delay in diagnosis or are never diagnosed. Endometriosis is characterized by the presence of endometrium (the inner lining of the uterus) in locations outside of the uterus. This endometrium then grows and forms lesions, termed ectopic endometrium or lesions [3]. This ectopic endometrium can be present on the ovaries, fallopian tubes, bowel, bladder, and in rare cases, the lungs and the brain [4] (**Figure 1).** The lesions present have also been known to respond in the same manner to steroid hormones as the eutopic endometrium. The levels of the estrogen receptor that are present in women with endometriosis are much higher than in women without endometriosis, while the progesterone receptor is at lower levels [5-7]. Some of the signs and symptoms that women with endometriosis experience include painful periods (dysmenorrhea), pain during intercourse (dyspareunia), pain in the pelvis, bloating, heavy and irregular bleeding, fatigue, and infertility [2]. In fact, many times it is when women are trying to conceive that they are diagnosed with endometriosis and it is one of the reasons as to why conception is difficult. It has also been stated that there can be an 8-12 year delay in actually getting a diagnosis of endometriosis from the first time symptoms are noted [8].

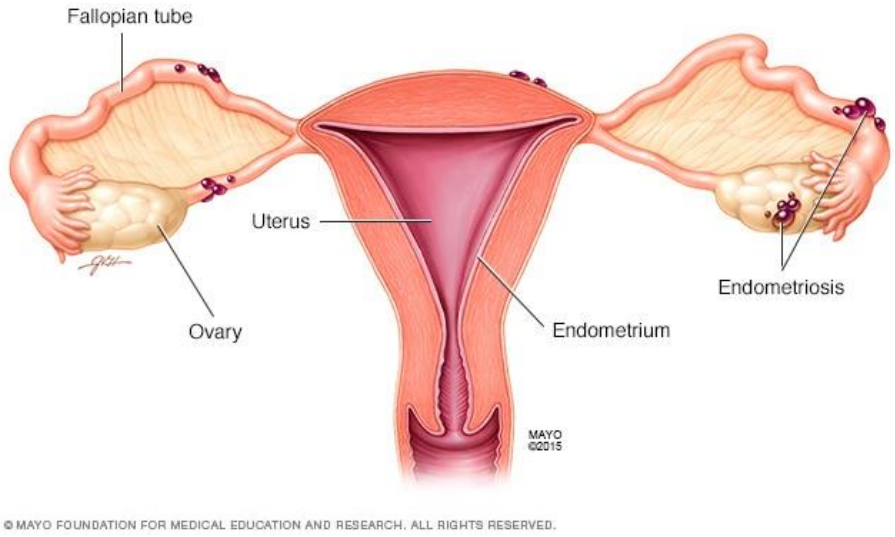

Figure 1: Uterus with lesions present in locations outside of the endometrium

Though the etiology of endometriosis still remains unknown, there are current theories that attempt to explain the formation of endometriosis. The first, and most widely accepted, is the "Sampson's theory" which was proposed in 1927 [9]. This theory is based on retrograde menstruation in which the endometrium that is shed during menstruation flows back into the abdominal cavity, where it can attach and grow. This in turn promotes a chronic inflammatory response [9]. The second theory is the "coelomic metaplasia theory" which states that endometriosis actually originates from specialized cells that are present in the mesothelial lining and that this *in situ* transformation of normal tissue to endometrium is stimulated by hormones or other factors [10,11]. The third most widely accepted theory is the "mulleriosis and embryonic origin theory". This theory states that endometriosis is due to developmental abnormalities in the female reproductive system, specifically the Mullerian duct system. It is also thought that endometriotic lesions are actually present throughout the whole development and maturation of women [12]. Another theory that is not as widely accepted is the "stem cell theory". This theory goes along with Sampson's theory in its hypothesis that the mesenchymal stem cells that line the

endometrium are shed during menstruation and flow back into the abdominal cavity, leading to lesion formation. It is also believed that it is the stem cells' ability to regenerate that leads to endometriosis [13]. There are also suggestions that endometriosis could be an autoimmune disease [14,15] and/or genetics playing a role, although no specific genetic link has been shown. This chapter will provide an overview of the role of inflammation in endometriosis, epigenetic mechanisms in endometriosis, and the possible interplay between inflammation and epigenetic mechanisms. This chapter will also highlight, current and potential future targets for the treatment of endometriosis.

ROLE OF INFLAMMATION AND OXIDATIVE STRESS IN ENDOMETRIOSIS

One thing that is common among all suggested theories is that endometriosis is a highly inflammatory disorder. This inflammatory environment and response has been well studied by our laboratory as well as others [16-25]. The peritoneal microenvironment in women with endometriosis plays a dynamic role in the disease etiology [26]. The peritoneal fluid (PF) in the abdominal cavity of women with endometriosis is one of the main sources of this inflammatory environment. The PF of women with endometriosis has been shown to be in larger quantities compared to women without endometriosis. The components of the PF include inflammatory cells, pain-inducing molecules, growth factors, and cytokines/chemokines, just to name a few, which are also altered in women with endometriosis [17,24,27].

The lymphokines that are present in the PF, whose main function is to regulate proliferation and differentiation of lymphocytes, can also stimulate monocytes that are present in the bloodstream to enter the peritoneal cavity and transform into activated macrophages. These peritoneal macrophages, either already present or recently transformed from the newly recruited monocytes, are involved in immune surveillance. Macrophages are white blood cells in the

immune system that digest cellular debris and foreign substances such as cancer cells. However, peritoneal macrophages in women with endometriosis have been shown to have lower phagocytic activity [28,29]. Macrophages are also known to be pro-inflammatory due to their secretion of growth factors such as TNFα, IL-1, IL-6, and IL-18 [30].

Tumor necrosis factor α, TNF-α, is a cytokine that has been shown to promote proliferation, adhesion, and angiogenesis of endometrial cells. It is produced by the lesions themselves and released into the PF [31]. The TNF-α signaling pathway has been shown to link inflammation and cancer as well as causing cells to obtain anti-apoptotic properties and become resistant to antigrowth signals [32,33]. IL-6 is upregulated in the PF and has been shown to cause an increase in ICAM (intercellular adhesion molecule). ICAMs are also upregulated in the ectopic endometrium and PF with these higher concentrations having been hypothesized to possibly play a part in cells evading the immune surveillance by affecting the immune response [34,35]. IL-8 is a cytokine that is unique to humans. IL-8 has been noted to be higher not only in the PF, but also in the ectopic endometriotic lesions where it can cause migration of neutrophils, which also play a role in inflammation [36,37]. IL-4 is another interleukin that is high in the PF of women with endometriosis. This high expression is thought to be another mechanism for the macrophage accumulation [38].

Matrix metallopeptidase (MMP-9) is part of a family of metalloproteinases that are involved in the degradation of the extracellular matrix. It has been shown to be expressed by inflammatory cells and to regulate inflammation by regulating the physical barriers, modulating cytokines and chemokines, as well as establishing chemokine gradients to regulate the movement of leukocytes to places of tissue injury [39]. A study by Collette et al. showed that MMP-9 concentrations are significantly higher in the tissues of women with endometriosis when

compared to normal controls. This increase in MMP-9 may enhance IL-1 activation of endometrial cells further contributing to inflammation and tissue remodeling in endometriosis patients. The authors stated that their findings in this study, along with their previous findings, support a role for MMP-9 in the invasion and development of endometrial tissues on a host tissue[40].

Vascular endothelial growth factor (VEGF) is a signaling protein whose function is to promote the growth of new blood vessels by acting on nearby endothelial cells. When VEGF is increased, this in turn increases the vascularity at sites of inflammation and causes the inflammatory reaction to be increased. It has been shown that when human vascular endothelial cells were treated with VEGF, it resulted in an increase in production of fibroblast growth factor, IL-8, and MMP-9, all of which have been shown to be increased in endometriosis as mentioned above [41]. VEGF expression is higher in the ectopic endometrium when compared to eutopic and peritoneum, increased in the PF of women with endometriosis as well as produced by the peritoneal macrophages present in endometriosis [42-44].

Our laboratory has also shown that the scavenger function of activated monocytes is compromised in endometriosis, however, they can still secrete cytokines and growth factors [21,45]. Once macrophages from PF from women with endometriosis gain scavenger activity, they can become adherent as well as able to take up and degrade oxidatively modified lipoproteins even more effectively than macrophages from women without endometriosis [46,47]. Recently, Forster, et al. treated a mouse model of endometriosis with liposomal clodronate, which lead to the depletion of large peritoneal macrophages (LpMs), monocyte-derived macrophages (MDMs), and an increase in the number of monocytes present. They also noted behavior differences in these mice when compared to non-treated mice, such as less spontaneous grooming and

increased pain withdrawal thresholds such as paw withdrawal. The authors concluded that it appears that macrophages and macrophage-derived mediators may activate changes in sensory behavior. They also saw in this study that the mice with endometriosis had an increase in TNF-α and that those levels were reduced when macrophages were depleted. The final finding they noted in this study was that there was an increase in *IGF-1* mRNA expression in the mice with endometriosis when using PF from women with endometriosis and pain to model endometriosis-associated macrophages (EAMs). This increase in expression correlated with increased levels of pain in these women as well as enhanced sprouting of the dorsal root ganglion (DRG) neurons which increased nociception or triggering of nociceptive nerve fibers due to inflammation or tissue damage. By inhibiting IGF-1, pain behavior was shown to be higher vs non-inhibited mice further indicating that pain mechanisms may be caused by IGF-1. Thus concluding that macrophages, such as the ones present in the PF of women with endometriosis, may be good targets for therapy by reducing the pain that these women experience [29,48]. **(Figure 2).**

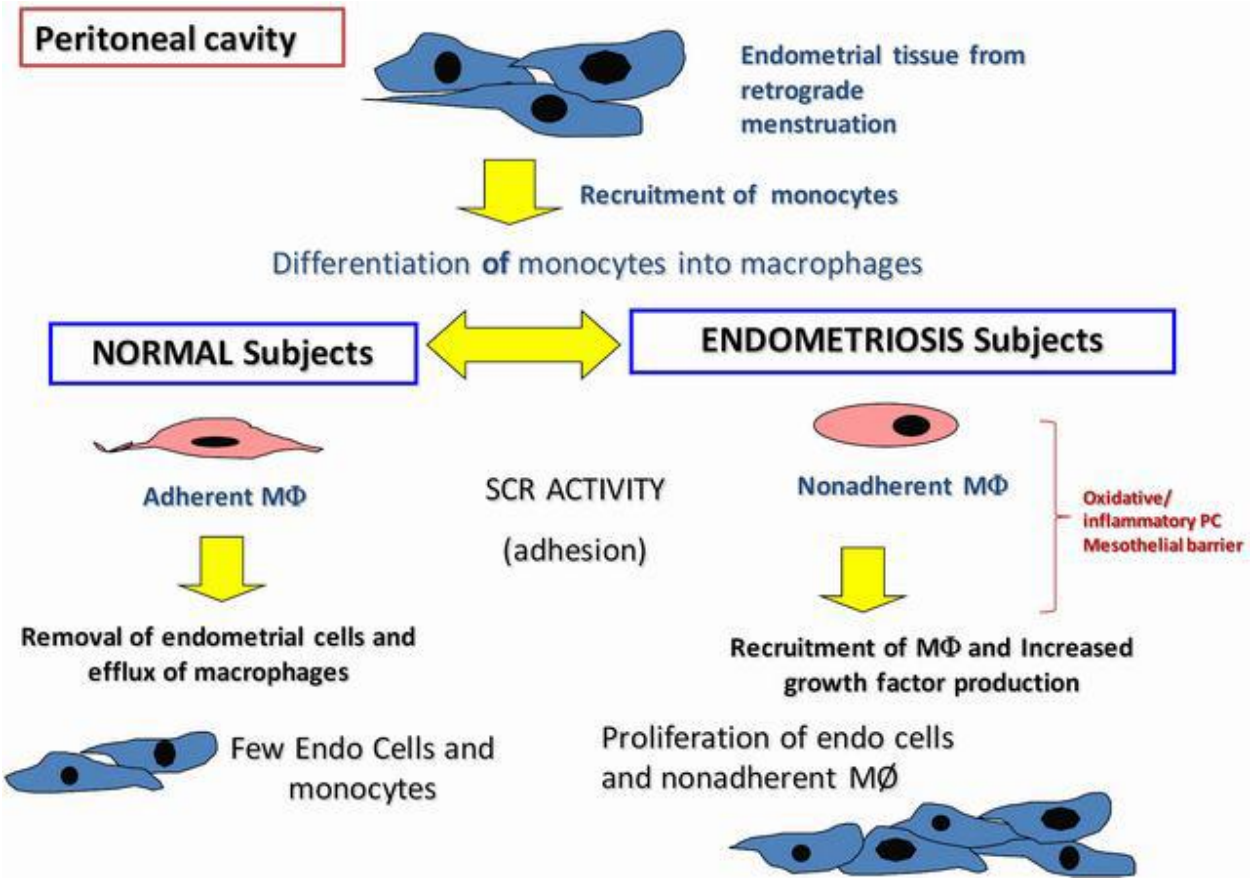

Figure 2: Schematic representation of the macrophage (dys)function in endometriosis SCR, scavenger receptor; PC, peritoneal cavity [29].

The production of interleukins and other growth factors due to the macrophages that are present in the peritoneal cavity is considered the primary mediator of the inflammatory response that occurs. It is also thought that these angiogenic and other growth factors can contribute to the development of vasculature of endometriotic explants, as well as their maintenance [49]. One such growth factor is transforming growth factor-β (TGF-β), which has been shown to control cell growth, proliferation, and differentiation along with apoptosis. Research has shown that the levels of this growth factor increase in the PF of women with endometriosis as the stage and severity of the disease increase [50].

Prostaglandins (PGE2 and PGF2 alpha) are also present in large quantities in the peritoneal cavity and fluid. PGE2 has been shown to be produced by macrophages and the lesions that are present. PGE2 has also been shown to increase estrogen synthesis by upregulating aromatase as well as promoting angiogenesis by upregulating VEGF [51]. The amount of prostaglandin F2 alpha (PGF2 alpha) that is present is also thought to contribute to the pain that these women experience due to the fact that it has been shown to increase the constriction of the uterus and the intensity of the constrictions [52]. All of these components mentioned above are also thought to play a role in the infertility that women with endometriosis may experience, as well as being shown to induce an inflammatory response and endometriotic lesion growth in animal models of endometriosis [20,53,54].

We have also shown the increased presence of oxidative stress markers in the PF, how they differ from the women without endometriosis, and how they play a role in the etiology of endometriosis [16,21,24,25,55]. Our laboratory hypothesized that oxidatively-modified lipoproteins are one of the main sources of nociceptive molecules that are involved in endometriosis-associated pain. An increased presence of prostaglandins that were derived enzymatically, as well as oxidized lipids, were increased in the PF of women with endometriosis. It was noted that 90% of endo PF was positive for the presence of lipoproteins, either LDL (low density lipoproteins) or HDL (high density lipoproteins), in contrast to only 45% positive in control PF. Using a Hargreaves paw withdrawal assay, a nociceptive response was noted when oxidatively modified LDL was injected into the paw. This response was also seen when PF from women with endometriosis was injected. These results supported the potential role of oxidized-lipid molecules present in the PF in the pain that women with endometriosis experience [16,17].

Table 1 summarizes the inflammatory molecules that have been noted to be upregulated in endometriosis and discussed above.

The studies that have been performed examining the levels and the effects of these molecules that are present in the PF of women with endometriosis help point to a contribution in the increase in inflammation and the pain that they experience in comparison to women without endometriosis, as well as the possible pathophysiology behind this disease.

Name	Abbreviation	In endometriosis
Tumor necrosis factor α	TNF-α	Higher concentrations, promotes proliferation of endometrial cells [31]
Interleukin 6	IL-6	Increased in PF, induces ICAMs [34]
Intercellular adhesion molecule	ICAM	Higher in ectopic tissues and PF [35]
Interleukin 8	IL-8	Increased in PF and ectopic lesions [37]
Interleukin 4	IL-4	High concentrations in PF [38]
Matrix metallopeptidase	MMP-9	Upregulated in endometrial tissues in women with endometriosis [40]
Interleukin 1	IL-1	Enhanced by MMP-9, contributes to further inflammation [40]
Vascular Endothelial Growth Factor	VEGF	Increased in ectopic lesion and in the PF [42,44]
Transforming Growth Factor β	TGF-β	Increased concentrations in the PF [50]
Prostaglandin E2	PGE2	Increases estrogen synthesis and upregulates VEGF [51]
Prostaglandin F2	PGF2	Large quantities in the PF. May contribute to infertility [52]
Low or High Density Lipoprotein	LDL or HDL	Increased amounts in the PF of women with endometriosis [16,17]

Table 1: Inflammatory and oxidative stress markers noted to be playing a role in endometriosis

EPIGENETICS

Epigenetics refers to nongenetic influences on gene expression that can arise in an organism. These reversible and dynamic changes that affect a gene's phenotype do not change the DNA sequence or content and can be modified by environmental or lifestyle factors. There are three different epigenetic mechanisms: DNA methylation, histone modifications, and microRNA [56,57].

DNA methylation is the most common epigenetic mechanism of the three known. This mechanism can regulate many cellular processes such as transcription, embryonic development, and chromatin structure and stability, just to name a few [58]. The first half of the methylation process is the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA through the use of DNA methyltransferases (DNMTs). The second half of the process utilizes methyl-CpG binding proteins (MDBs). These MDBs work to recognize and read the methylation marks that have been made by the DNMTs. When this binding occurs, the result is typically loss of gene expression or gene silencing due to the methylation of the CpG islands inhibiting transcription factors from binding [58] and can be passed down to daughter cells [59,60]. This work by the DNMT enzymes is also thought of as "on-the-spot DNA methylation" completing the chromatin in that closed or heterochromatin state [61,62] **(Figure 3A).**

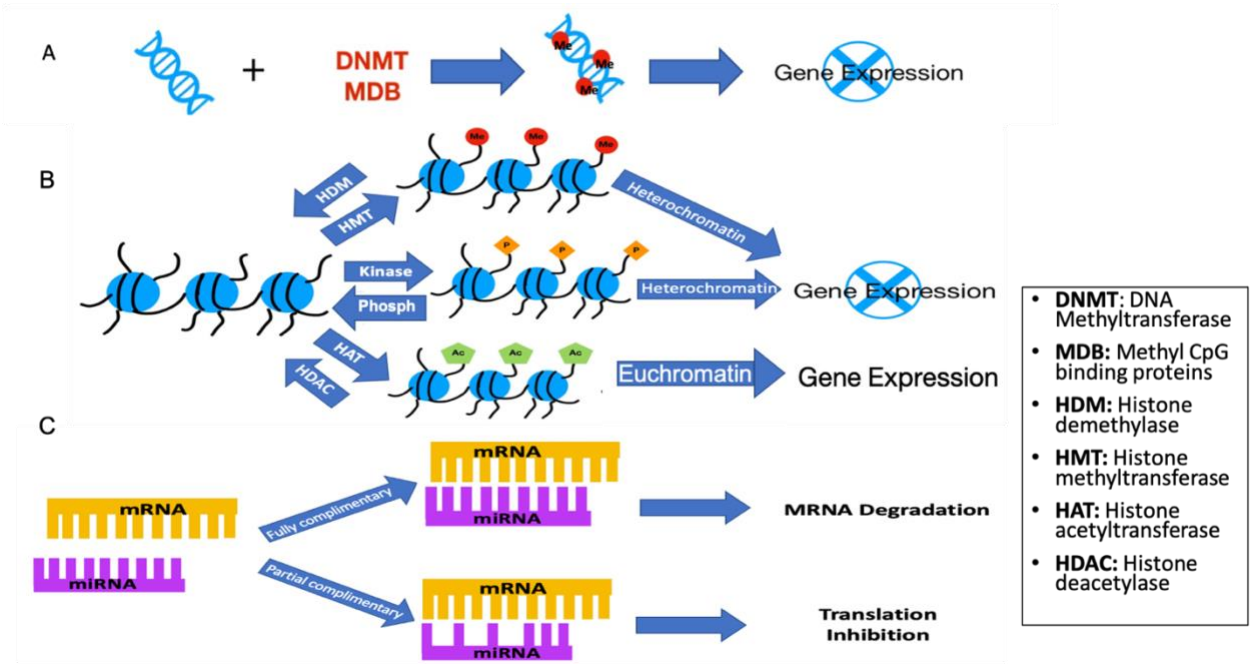

Figure 3: The three types of epigenetic mechanisms: A. DNA methylation B. Histone modifications C. microRNA [63].

Histone modifications are the second common type of epigenetic changes and are shown to occur post-translationally (commonly referred to as PTMs, post-translational modifications). Once these modifications occur, the chromatin structure, as well as the recruitment of modeling enzymes, is affected. There are three common ways that histone modifications can occur. The first and most widely discussed is methylation. Methylation occurs on the lysines and arginines on the histone tails through histone methyltransferase (HMT) enzymes and can be reversed through the use of lysine demethylase I (LSD1). Histone modifications can also occur through phosphorylation which can take place on serines, threonines, and tyrosines. Phosphorylation adds a negative charge to the tail, changing the conformation of the chromatin. Phosphorylation also causes the formation of heterochromatin and subsequent shutting down of gene transcription. The third type of histone modification is acetylation. This modification occurs on the lysines of the tail through the use of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation modifications differ from methylation and phosphorylation in that when a histone is acetylated, this actually forms euchromatin and gene transcription is allowed [64,65]. Modifications made by chromatin remodeling have been shown to be at a higher level of diversity and complexity than the modifications made by DNA methylation due to the changes affecting the chromatin structure which influences the gene expression. The expression of the genes can also be affected by chemical modifications that occur on the histone proteins [66] **(Figure 3B)**.

The Polycomb repressive complex 2 (PRC2) is a key regulatory complex that is involved in histone modification, specifically through its major subunit, Enhancer of Zeste Homologue 2 (EZH2). EZH2 works as a histone methyltransferase, playing a key role in transcriptional repression through chromatin remodeling by activating H3K27 through trimethylation, forming H3K27me3 [67-71]. Studies have shown that there are elevated EZH2 expression levels in various

cancers [69,72,73]. AEBP2 is a zinc-finger protein that works with the PRC2 complex to stabilize its structure and stimulate PRC2 activity [74,75]. Jumonji and AT-rich Interaction Domain Containing 2 (JARID2) is a member of a family of histone demethylases, the jumonji family, but lacks a histone demethylase domain [76,77]. JARID2 is also a known cofactor for the PRC2 complex [78]. JARID2 works by bringing the complex to its target (H3K27me3) and may help increase the complex's catalytic rate [75] **(Figure 4).**

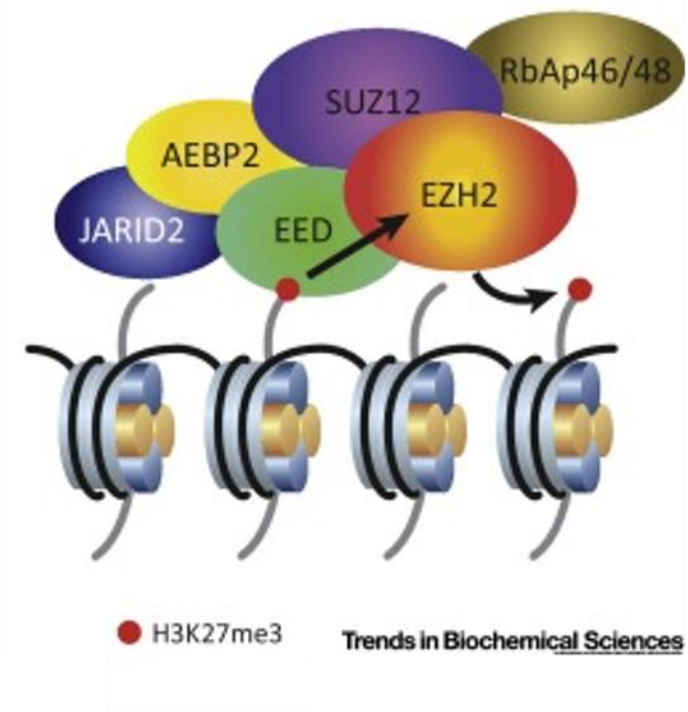

Figure 4: PRC2 complex and its regulators [75]

The third, and last type of epigenetic modification is through microRNAs (miRNAs). miRNAs are non-coding, single-stranded RNAs and usually consist of only 22 nucleotides. They perform gene regulation and RNA silencing, without changing the DNA sequence, through two different mechanisms. The first is if the miRNA is completely complementary to the mRNA target sequence, then mRNA degradation takes place. The second is if the mRNA is partially

complementary to the target mRNA sequence. When this occurs, the mRNA is prevented from being transcribed **(Figure 3C)**. DNMTs, HDACs, and the PRC2 complex are all controlled by several miRNAs, forming an epigenetic-miRNA regulatory circuit and feedback network [79,80].

EPIGENETICS IN ENDOMETRIOSIS

While the proposed theories for formation for endometriosis mentioned above encompass some aspects of endometriosis, none have been shown to fully explain the etiology of this disease. Many researchers are investigating if epigenetic mechanisms may be playing a role in the initiation and progression of endometriosis. Three DNMTs (DNMT 1, 3A, and 3B) are overexpressed in ectopic tissues compared to eutopic tissues from women with endometriosis [81]. Hypomethylation of the ERβ promoter leads to an increase in its mRNA expression, as well as hypermethylation of PR-B [82,83]. These epigenetic mechanisms are likely why there is an imbalance in the estrogen and progesterone levels in women with endometriosis. Another gene that is downregulated due to hypermethylation at its promoter region is E-cadherin, which is important in cell-cell adhesion [84]. A study by Zhu, et al. showed that when E-cadherin was knocked down in endometrial epithelial cells (EECs) from normal endometrium, there was an increase in cell migration and invasion, similar to what is seen in endometriosis patients [85]. HOXA10, a highly conserved homeobox gene, and its promoter have also been noted to be hypermethylated in endometriosis. This is important as this gene is known to serve as a transcription factor, as well as being important for uterine function. This may be one reason why uterine receptivity is reduced in endometriosis patients [56,86]. In addition, PR downregulation due to hypermethylation may have an impact on the lower expression of HOXA10 [87].

Histone deacetylases (HDACs), histone acetylaces (HATs), histone methyltransferases (HMTs), and the methyl-CpG-binding protein MeCP2 have been shown to be involved in histone modifications that occur in endometriosis [56]. One of the most studied regulatory complexes in endometriosis is the PRC2 complex, as described above. It has been noted that EZH2 can enhance the epithelial-mesenchymal transition in endometriosis [88]. Our laboratory, and later confirmed by Colon-Carballo [89], showed an increased expression of EZH2/H3K27me3 in endometriotic lesions [90-93]. Histone levels in endometriosis have also been shown to vary from women without endometriosis, with higher methylation levels of H3K4 and H3K9 and lower acetylation levels of H3K9 and H4K16 [92].

MicroRNAs are an emerging topic of interest in endometriosis, including in our own research, due to their role in epigenetic regulation [94]. Many studies have shown the profiles of not only the miRNAs that are present in the tissues of endometriosis patients but also the circulating miRNAs and how they differ from women without endometriosis. Specifically, one study showed a reduced expression of 48 differentially expressed miRNAs in eutopic and ectopic tissue when compared to control tissues. miR-21 and miR26a were specifically shown to be dysregulated [95]. miR-141 is another microRNA that has been seen to be decreased in expression in the ectopic tissues when compared to eutopic or control tissues. One specific study showed that endometrial cells had an increase in cell proliferation, but a decrease in migration when transfected with miR-141. These transfected cells also showed a decrease in the oncogene Kruppel like factor 12 (KFL-12), which suggests that it is a direct target of miR-141 [96]. miR-214 is involved in pro-growth and anti-apoptotic pathways and targets the tumor suppressor Phosphotase and tensin homolog (PTEN). A study by Lv et al. showed that when PTEN levels were increased in a mouse model of endometriosis, increased apoptosis and inhibition of cell

cycle progression was seen when compared to control or silenced PTEN groups [97]. It has also been noted that EZH2 is increased when miR-214 is downregulated suggesting that miR-214 targets EZH2 [98]. **Figure 5** summarizes the epigenetic mechanisms seen in endometriosis.

Figure 5: List of epigenetics marks that have been identified in endometriosis and discussed

INTERPLAY BETWEEN INFLAMMATION AND EPIGENETICS IN ENDOMETRIOSIS

With the information presented above of how inflammation and epigenetics are independently playing a role in the disease progression of endometriosis, it is no surprise to assume that inflammation is having an effect on the epigenetic mechanisms at play in endometriosis, and vice-versa. In fact, it has been stated that distinct, global changes in the epigenetic landscape are a hallmark of chronic inflammation-driven diseases, like endometriosis [99]. Our laboratory has specifically highlighted that link when showing that miRNAs such as miR-29a and let-7g, oxidative sensitive microRNAs, may be playing a role in endometriosis associated pain [17].

Studies have shown that TNFα, highly expressed in the PF of women with endometriosis, is able to induce partial methylation of the promoter region of PR-B in endometriotic cells, causing a decrease in the levels of progesterone present [83,100]. TGFβ, as mentioned above, has

been shown to be higher in women who have been afflicted with endometriosis for a longer period of time. A recent study demonstrated that the low expression of miR-141 drove the TGFβ1/SMAD signaling, activating EMT in endometriosis. miR-141 transfection led to the activation of TGFβ1 and induction of EMT [101]. This is important when discussing the interplay between epigenetics and inflammation as TGF-β, in the presence of IL-6, can promote inflammation by driving the differentiation of the T helper 17 cell (Th17) [102]. Th17 cells can also produce other interleukins such as IL-21 and IL-22 whose receptors are present on epithelial tissues mediating a cross-talk between the immune system and the epithelial tissues [103]. Studies have also found that alteration of the TH17 cells in the pelvis allows for survival and implantation of the ectopic endometrial lesions [104].

Steroidogenic factor-1, SF-1, is a nuclear receptor known to regulate many genes in the hypothalamic-pituitary-adrenal endocrine axis. In endometriosis, increased expression of SF-1 is due to the hypomethylation of the CpG islands. SF-1 is recruited to the promoter of steroidogenic genes and in turn promotes estradiol synthesis, essential for the growth and inflammation of the endometriotic tissues [82,105-108]. miR-23a and miR-2b have been shown to be suppressed in the endometrium of women with endometriosis, which allows for the high expression of SF-1 [109,110]. This also furthers the expression of CYP19A1 (aromatase), an enzyme that controls the key step in estrogen biosynthesis [111,112].

Cyclooxygenase-2, COX-2, is an enzyme that is usually not a factor in normal systems, but it is highly expressed in endometriosis. This is due to the high number of cytokines and proinflammatory agents present stimulating its expression, specifically IL1β and TNF-α [113,114]. It has also been seen that there is an increase in COX-2 in endometriosis due to the downregulation of miR-199a and miR-16 [115]. COX-2 has also been noted to be upregulated due to aromatase

activity and the subsequent stimulation of prostaglandin E2 (PGE2). PGE2 is known to be the primary mediator of inflammation and hypersensitivity to pain in women with endometriosis. Increased COX2 results in an increase in PGE2 levels in the PF these women [116].

Reports have shown that there is a decrease in global methylation levels in ectopic stromal cells in endometriosis due to hypoxia mediated DNMT1 downregulation. Studies have also implied that the levels of DNMT3a may be maintained or stimulated by inflammation pathways [117-119].

Sirtuin 1, SIRT1, is an NAD-dependent histone deacetylase which plays a role in the regulation of inflammation. It has been shown to inhibit TNF-α-induced inflammation as well as to inhibit the activity of NF-κB [120,121]. SIRT1 can also target progesterone-related genes. Studies have shown that SIRT1 is highly expressed in the endometrium of patients with endometriosis and plays a role in progesterone decrease [122]. However, other studies have shown that a SIRT1 activator, resveratrol, was beneficial in endometriosis by suppressing TNF-α and IL-8 release [123]. More work is needed to fully uncover the role of this HDAC in endometriosis.

CURRENT TREATMENTS

Inflammation and pain targeting treatments

Currently, treatments for endometriosis only treat the symptoms associated with the disease, such as the pain and the inflammation, and not the disease itself. Many of these are hormonal treatments as it has been shown throughout this chapter that endometriosis is a hormonal disease with an imbalance in hormone levels. Oral contraceptives/birth control pills are commonly used due to their ability to lower estrogen levels in the endometrium and endometriomas, as well as their ability to block ovulation [124]. As long as the patient is taking the

active contraceptive pills, the pain levels remain decreased. However, once the pills are stopped, the pain returns [125]. Progestins are also a form of treatment to increase the low levels of progesterone seen in women with endometriosis. These drugs can be through oral ingestion, via an intrauterine device (such as Mirena and Kyleena), or by depot injection [126]. While these can be effective in pain relief, breakthrough bleeding can occur, which in turn causes pain.

Aromatase inhibitors, such as Arimidex, Aromasin, and Letrozole have also been used. As mentioned earlier, CYP19A1, aromatase, is the key enzyme in estrogen biosynthesis. The use of these inhibitors has been shown to decrease pain, as well as delay reoccurrence rates after women who had surgery [125]. These inhibitors have also been used in combination treatment with birth control pills or progestins [127-129].

NSAIDs (non-steroidal anti-inflammatory drugs) work by blocking COX-2 which is highly expressed in endometriosis due to the high number of inflammatory molecules present in the PF. NSAIDs are the most frequently prescribed drug for endometriosis associated pain [130]. Over-the-counter NSAIDs include ibuprofen (Motrin, Advil), aspirin, and naproxen (Aleve). For NSAIDs to work effectively in women with endometriosis, it should be taken 24 to 48 hours before ovulation begins, as well as before the first day of their period. This ensures that the medication has time to block the production of prostaglandins in the body. Just like aromatase inhibitors, NSAIDs are normally used in combination with other therapies [131].

Gonadotropin-releasing hormone (GnRH) agonists, such as the Lupron Depot shot, are also a form of pain-relieving treatment for endometriosis. It is a modified version of GnRH, a naturally occurring hormone that works to control the menstrual cycle. They can come in different forms such as a once every three months injection, once monthly injection, daily injection, and a nasal spray. GnRH agonists work by depleting the estrogen that is present.

However, they have many side-effects associated with it, such as inducing a menopause-like state and bone thinning [132]. A prescription GnRH antagonist medication called Orilissa was introduced into the market in July 2018 and is specifically marketed to reduce endometriosis associated pain. Orilissa has been shown to reduce the symptoms of dysmenorrhea (painful menstruation) in 43-72% of women with endometriosis as well as reducing pelvic pain in 50-58% of women [133,134].

Laparoscopic surgery is also a common practice in endometriosis. This can either be for diagnosis or for treatment where the lesions are removed. The PF that is present is also removed during surgery. Both the removal of the lesions and the PF reduce the pain associated with endometriosis as many of the inflammatory molecules are being removed in the process [135]. However, surgery is not an end-all treatment as lesions have been shown to grow back within just a few months [136]. Many women with endometriosis are also turning towards getting a hysterectomy for relief. This is still not a completely successful treatment as lesions have been shown to return in these women [137].

Trichostatin A, TSA, a compound that selectively inhibits histone deacetylase enzymes, has begun to be tested in endometriosis [138]. Specifically, researchers are looking at the effects that it has on nonsteroidal anti-inflammatory drug activated-gene-1 (NAG-1) mediated apoptosis and expression. NAG-1 is a member of the TGF-β family and has been shown to be decreased in the endometrium of patients with endometriosis. Studies showed that the expression levels of NAG-1, as well as apoptosis, were increased when ectopic endometrial tissues were treated with TSA [139]. It has also been seen that the use of TSA can reduce the number and size of endometrial implants in mice [140].

New potential drugs possibly targeting epigenetic pathways

Targeting epigenetic mechanisms in endometriosis as a more tailored treatment has begun to be explored, much like in the same manner as it is being explored in cancer for "personalized medicine" [141,142]. The research in endometriosis, currently still only in pre-clinical trials, has focused on HMT inhibitors, specifically inhibiting the expression of EZH2. Research using 3-Deazaneplanocin A hydrochloride, DZNep, and GSK126 has been investigated. DZNep is a lysine methyltransferase EZH2 inhibitor and competitive s-adenosyl homocysteine hydrolase inhibitor which works to block H3K27me3 and H4K20me3 [143]. GSK126 is a selective, S-adenosyl-methionine (SAM)-competitive small molecule inhibitor of EZH2 methyltransferase activity which is more than 1,000-fold selective for EZH2 over other HMTs [144]. GSK126 is a newer compound on the market and has not been studied as extensively in endometriosis as DZNep has. DZNep was shown to inhibit the growth of endometriotic lesions, along with reducing EMT and fibrosis *in vivo* [88]. GSK126 has been noted to suppress endometrial cancer progression by decreasing the levels of EZH2 and increasing the levels of the tumor suppressor miR-361 [145]. GSK343 is another SAM competitive inhibitor that has been explored in endometriosis. Researchers noted a decrease in H3K27me3 levels, as well as decreased cell proliferation with the use of GSK343 on endometriotic cells [93].

Valproic acid, VPA, works through generation of reactive oxygen species to inhibit HDACs. VPA was shown to inhibit cell cycle arrest and p21 expression in endometrial cells [146]. Also, using a rat model of endometriosis, VPA, in combination with progesterone, showed a reduced lesion size and increased body weight when compared to that of untreated rats. It was shown to reduce pain response suggesting that this combination may be an effective treatment in endometriosis [147].

CONCLUSIONS

Increases in not only the inflammation and oxidative stress molecules present, but also the epigenetic mechanisms and changes due to those mechanisms, have been observed in women with endometriosis when compared to women without. Connections between the inflammation present and those epigenetic mechanisms have also been shown in endometriosis. However, the question still remains about the driver behind it all. Are the epigenetic mechanisms initiating events which subsequently increase the inflammation or is the high inflammation activating the epigenetic mechanisms in endometriosis? Could it be possible that they are affecting each other equivalently, making for an even more complex disease and therefore a complex treatment? The objective of the research presented in this thesis was to further explore this epigenetic-inflammation interplay seen in endometriosis and to identify targets for treatment for endometriosis. We also wanted to explore if we could treat not only the pain associated with endometriosis, but also the lesions that are present by reducing their size. It is possible that reducing the size of the lesions would reduce the pain due to less lesion present to release inflammatory molecules into the peritoneal cavity. We hypothesized that the peritoneal fluid, and the inflammatory molecules that are present within this milieu, are driving the mechanisms leading to endometriosis, specifically the modulation of the PRC2 complex and EZH2. We also hypothesized that the histone methyltransferase activity of EZH2 can either be decreased or the activity completely inhibited by blocking inflammatory pathways. These hypotheses were tested through the use of patient tissues and cell models of endometriosis.

CHAPTER 2

PERITONEAL MODULATORS OF EZH2-MIR-155 CROSS-TALK IN ENDOMETRIOSIS

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ABSTRACT

Activation of trimethylation of histone 3 lysine 27 (H3K27me3) by EZH2, a component of the Polycomb repressive complex 2 (PRC2), is suggested to play a role in endometriosis. However, the mechanism by which this complex is dysregulated in endometriosis is not completely understood. Here, using eutopic and ectopic tissues, as well as peritoneal fluid (PF) from IRB-approved and consented patients with and without endometriosis, the expression of PRC2 complex components, JARID2, miR-155 (known regulators of EZH2), and a key inflammatory modulator, FOXP3, was measured. A higher expression of EZH2, H3K27me3, JARID2, and FOXP3 as well as miR-155 was noted in both the patient tissues and in endometrial PF treated cells. Gain-or-loss of function of miR-155 showed an effect on the PRC2 complex but had little effect on JARID2 expression, suggesting alternate pathways. Chromatin immunoprecipitation

followed by qPCR showed differential expression of PRC2 complex proteins and its associated binding partners in JARID2 vs EZH2 pull-down assays. In particular, endometriotic PF treatment increased the expression of *PHF19* (p=0.0474), a gene silencer and co-factor that promotes PRC2 interaction with its targets. Thus, these studies have identified the potential novel crosstalk between miR-155-PRC2 complex-JARID2 and PHF19 in endometriosis, providing an opportunity to test other epigenetic targets in endometriosis.

Keywords: endometriosis, epigenetics, EZH2, microRNA

INTRODUCTION

Endometriosis is defined by the presence of endometrial tissue in ectopic locations, typically in or around the peritoneal cavity [3,136]. While the exact prevalence of endometriosis is likely underrepresented, most sources cite that a minimum of 10% of women in their reproductive years have this disease [1,148,149]. Primarily described as a hormonal disorder, the pathogenesis of endometriosis has also been linked to immunological/inflammatory, genetic, and environmental factors. More recently, the role of epigenetics in the development and progression of this disorder has been investigated [56,63,150-154]. Epigenetic mechanisms are heritable changes to one's phenotype that are not associated with a change in nucleotide sequence and include DNA methylation, post-translational modifications to histone proteins, and often microRNAs [155,156].

In addition to heterochromatin-like protein 1 (HP-1), polycomb (PcG) and trithorax (TrxG) complexes are at the heart of epigenetics. Responsible for maintaining gene repression and activity, respectively [157], the latter two complexes function antagonistically to establish epigenetic regulation [157]. Polycomb repressive complex 1 (PRC1), polycomb repressive complex

2 (PRC2), and Pho repressive complex (PhoRC) all form the PcG complexes, with the former two typically being the subject of extensive epigenetic research. The Polycomb Repressive Complex 2 (PRC2) consists of four core proteins, RbAp46/48, Embryonic Ectoderm Development (EED), Suppressor of Zeste 12 (SUZ12), and Enhancer of Zeste Homolog 2 (EZH2), the catalytic subunit of the PRC2 complex. These components work together to regulate chromatin structure via tri-methylation of lysine 27 on histone 3 (H3K27me3) [158,159], which is also known to interact with PRC1. EED binds the histone site while EZH2 methylates it, with the help of SUZ12 [160]. This modification leads to the formation of closed chromatin structure (heterochromatin) and thus marks transcriptional repression, as further demonstrated by the presence of other co-factors [161-163].

There is very little known about the mechanistic role of PRC2 complex and how it is regulated in the endometriosis disease process. While an in vivo study showed heightened expression of EZH2 and trimethylation of H3K27 in secretory endometrium and endometriotic lesions [93,164], another cell culture study showed that inhibition of PGE2 receptors EP3 and EP4 occur concurrently with decreased EZH2 expression [118], supporting a role for PRC2 in endometriosis-associated pain.

It has been shown that the PRC2 complex (specifically EZH2) is, at least partly, regulated by Jumonji and AT-Rich Interaction Domain Containing 2 (JARID2) [165], a member of the largest family of histone demethylases, the jumonji family, where all but JARID2 contain the catalytic JmjC domain responsible for histone demethylation [76,77]. Research has found that JARID2 is a cofactor for PRC2 [78]. Additionally, its methylation by the PRC2 complex at K116 is part of a regulatory mechanism that controls the PRC2 enzymatic activity where the methylated JARID2 binds to the EED component of the PRC2 complex. This is required for efficient deposition of

H3K27me3 during cell differentiation and fine-tunes the PRC2 activity [166]. JARID2 is thought to be crucial in the development and progression of cancer. This is due to its cross-talk with EZH2 and PRC2 activity in embryonic stem cells (ESC), as JARID2 is necessary for proper ESC differentiation [165,167,168].

JARID2 is suggested to be modulated by few mechanisms. For example, iron oxidation, which occurs due to increased reactive oxygen species generation and is known to be present in excess in women with endometriosis, blocks the catalytic activity of JARID2 [169]. JARID2 is also a common target of microRNAs some of which have been identified by our laboratory to be differentially expressed (miR-30b, miR-30c, miR-10a, miR-29a, miR-26a, miR-148a, miR-181a, miR-30e) in endometriotic lesions compared to control tissues [17]. Palma et al showed in acute lymphoid leukemia that miR-155-5p induced cell death via a network of mechanisms, including regulation of cyclinD1 by JARID2 [170]. Other such studies support the possibility that miR-155-5p could have been evolved to regulate PRC2 by tweaking JARID2 expression [171]. Interestingly, miR-155-5p is an established promoter of inflammation via regulation of macrophages and cytokines [171-173]. Thus, targeting this demethylase (JARID2) via modulators such as microRNAs could be a novel method of treatment for endometriosis.

miR-155 is highly expressed in regulatory T-cells (Tregs), where it is targeted by transcription factor forkhead box P3 (FOXP3) [174]. Though limited in evidence, FOXP3 also plays a role in the inflammatory aspect of endometriosis, which correlates with miR-155-5p being a promoter of inflammation. The prevalence of $FOXP3^{+}$ Tregs in an endometriotic environment during the secretory phase prevents leukocyte recruitment to the sites of endometriosis [175]. Additionally, peritoneal fluid (PF) from women with endometriosis has a

higher concentration of FOXP3-expressing TCD4^{+}CD25^{high} cells than the PF of control patients [176,177]. Other studies have also shown that FOXP3 is an inducer of miR-155 [178].

It is important to note that FOXP3 also has an indirect relationship with the EZH2 component of PRC2. Overexpression of the FOXP3 protein not only lessened the proliferative effects of EZH2, but also enhanced degradation of the EZH2 protein in breast cancer models [179]. Conversely, there is evidence that trimethylation of H3K27 by EZH2 is capable of silencing FOXP3 promoter regions, therefore leading to aberrant Treg cell differentiation and function [180]. These studies suggest a complex interplay between epigenetic mediators, PRC2 complex, miR-155-5p, JARID2, and the inflammatory mediator FOXP3. In this study, it is hypothesized that the imbalance in this crosstalk triggers inflammatory responses and possibly nociception in endometriosis. This current study investigated the crosstalk between these mediators in endometriotic patient tissues and in an endometriosis cell model.

RESULTS

PRC2 Complex and JARID2 mRNA and Protein Expression in Endometriotic Tissues

The endogenous expression of PRC2 complex proteins in endometriotic tissues was first determined. qPCR was used to determine the mRNA expression of PRC2 components SUZ12, EED, and EZH2 in eutopic tissue from women with no endometriosis (EuN, *n*=5) or women with endometriosis (EuE, *n*=10) and ectopic tissue from women with endometriosis (EcE, *n*=6) **(Figure 6A)**. When compared to the EuN tissues, expression of all three PRC2 complex proteins (*SUZ12*, *EED* and *EZH2*) and *JARID2*, was higher in both the eutopic (EuE) and ectopic (EcE) tissue from endometriosis patients. Compared to EuN tissues, *SUZ12* levels increased close to 2-fold for EcE but was not significant, however, there was a significant increase in *EED* expression

by 5.07-fold in EuE (p=0.0153) and 7.13-fold (p=0.0067) in EcE. *EZH2* expression was also increased 2.35-fold in the EuE and 3.10-fold in the EcE but did not reach significance. Expression for *JARID2* increased over 2-fold in EcE tissues, but this was not significant.

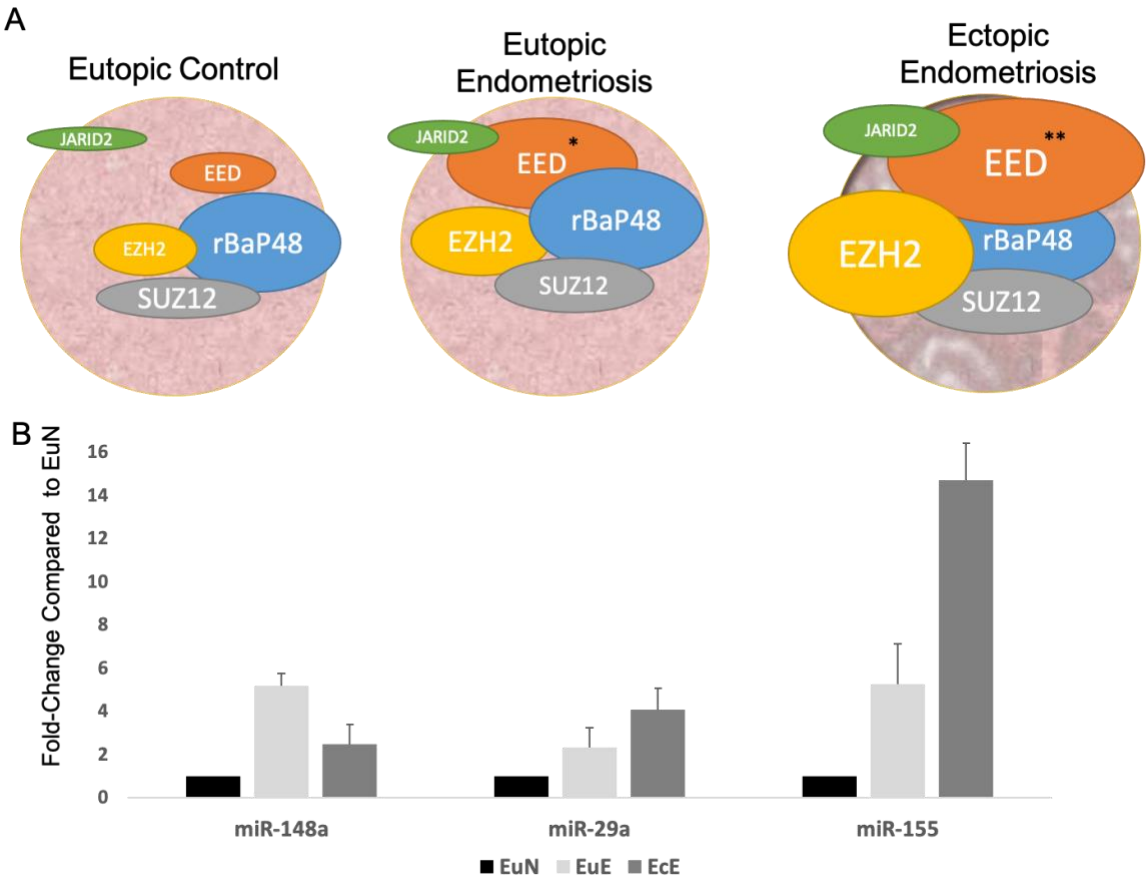


Figure 6: mRNA expression of PRC2 complex and JARID2 and miRNAs that target JARID2 in endometriotic tissues. **A)** Relative mRNA expression of polycomb repressor complex 2 (PRC2) elements and *JARID2* in eutopic tissues from control women, EuN (n=5), or eutopic and ectopic tissues from women with endometriosis, EuE (n=10) and EcE tissues (n=6). In general, these elements were upregulated in both eutopic and ectopic endo tissues compared to control tissue with *EED* showing significant upregulation in both the eutopic (p=0.0153) and ectopic (p=0.0067). *JARID2* expression was higher in EcE. *p<0.05, ** p<0.01 when compared to EuN tissues. **B)** Compared to control tissues (n=7), expression of miR-148a, miR-29a, and

miR-155 (miRNAs that target JARID2) were all higher in endo tissues (both eutopic and ectopic, *n*=8).

Protein expression was also determined using the automated Western blotting system, WES. While EZH2 showed a significant increase of >7 fold (*p*=0.0219) in EcE tissues compared to EuN, no significant difference was seen in expression of H3K27me3 or JARID2 (**Figure 7**). This lack of change in JARID2 expression might be attributed to its altered regulation.

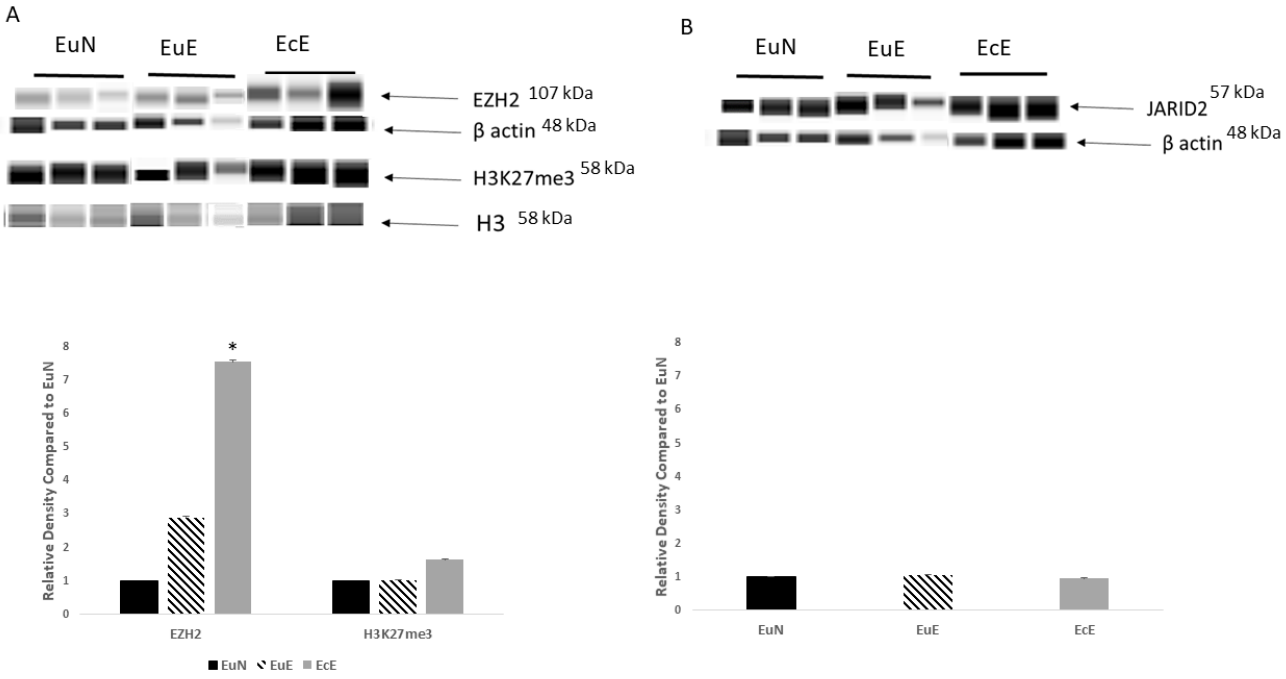


Figure 7: Protein expression of EZH2, H3k27me3 and JARID2 in patient tissues. A) WES images and densitometric analysis of protein bands of EZH2 and H3K27me3, in eutopic control (EuN), eutopic endo (EuE), and ectopic endo (EcE) tissues. A 7-fold increase in EZH2 expression was seen in EZH2 expression in EcE (*p*=0.0219) compared to EuN; **B)** WES images and densitometric analysis of JARID2 protein expression, in eutopic control (EuN), eutopic endo (EuE), and ectopic endo (EcE) tissues. Protein expression was calculated relative to β-actin for

EZH2 and JARID2; and H3 for H3K27me3. EuE and EcE were compared to EuN and shown as a ratio, where EuN was considered as 1.

miRNAs Targeting JARID2 in Endometriotic Tissues

The expression levels of miRNAs that regulate JARID2 were next determined in the patient tissues. miRNA qPCR assays were used to measure expression of miR-148a, miR-29a, and miR-155, which, among others, target JARID2 (Targetscan 7.1 and Ingenuity Pathway Analysis Qiagen, Germantown, MD, USA). Interestingly, all three miRNAs were overexpressed in both EuE and EcE tissues compared to EuN tissues **(Figure 6B)**. Both miR-148a and miR-155 showed an over 5-fold increase in expression for the EuE tissues and were also shown to be induced more than 2.5-14-fold respectively in EcE, while miR-29a expression increased 2-4-fold with levels higher in EuE and EcE tissues.

PRC2 Complex mRNA and Protein Expression in PF Treated Endometrial Cells

The peritoneal cavity is one of the major sites for endometriotic lesions in women with endometriosis [181,182]. These patients also exhibit larger volumes of PF rich in inflammatory and nociceptive molecules [183,184]. Current theories propose a dynamic role for PF in modulating the growth of endometriotic lesions, which might be epigenetically regulated by the altered expression of certain miRNAs previously shown in endometriosis [185,186]. Whether PF from patients with and without endometriosis differentially regulated the PRC2 complex proteins in endometrial cells was determined. For this, human endometrial cells were exposed to 1% PF from women with (*n*=13) or without endometriosis (*n*=12) for 48 h followed by the measurement of both mRNA and protein expression of PRC2 complex proteins using similar techniques as

described for the endometriotic tissues. Cells treated with both 1% control or endo PF had increased *SUZ12*, *EED*, and *EZH2* mRNA expression but none were shown to be statistically significant **(Figure 8A)**. When protein expression was determined using the automated Western Blotting system, WES, EZH2 showed no significant difference in expression levels when compared to the media control. While H3K27me3 did show an upregulation of over 2-fold for endo PF treated cells, this was not significant **(Figure 8B, C)**.

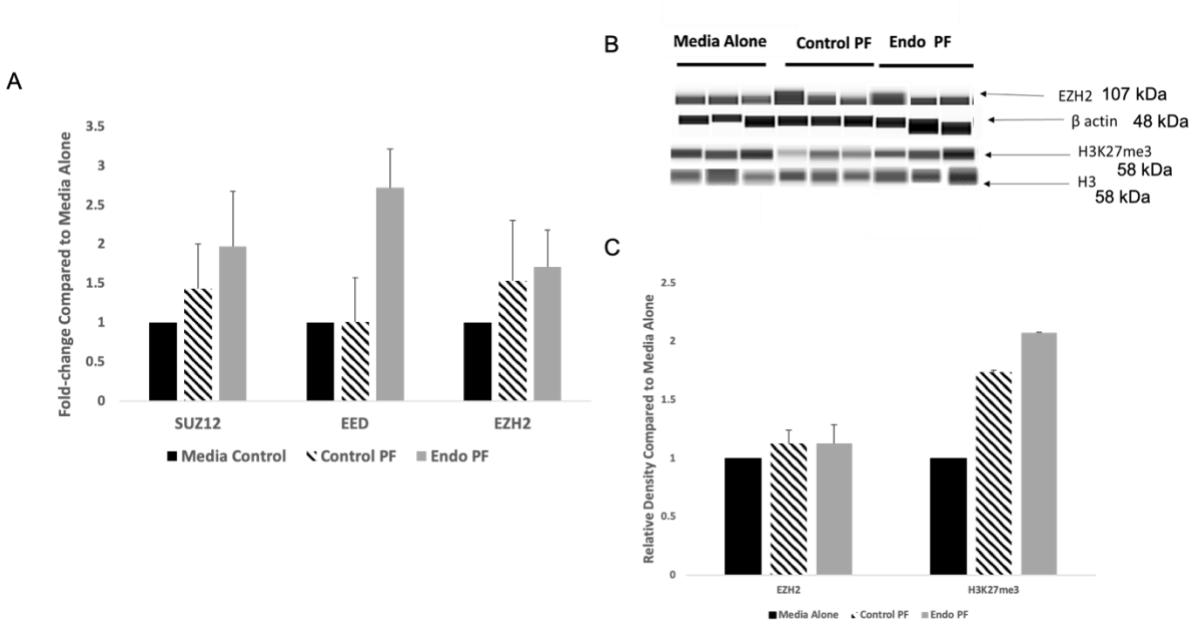


Figure 8: mRNA and protein expression of PRC2 complex proteins in PF treated endometrial cells. (**A)** mRNA expression of *SUZ12*, *EED*, and *EZH2* in cells treated with control PF (n=12) and endo PF (n=13), relative to expression in a media control (p>0.05). **(B)** Representative WES images and densitometric analysis for EZH2, and H3K27me3 in PF-treated cells. **(C)** Relative protein expression of EZH2, and H3K27me3 in PF-treated cells was calculated in relation to a media control and presented as a ratio in which media alone is 1. Densities of protein bands obtained were normalized to β-actin or H3. It is to be noted that

molecular weights of protein bands in the automated WES system differs from the traditional Western blotting, due to differences in technology.

JARID2 and miRNAs Targeting It in PF Treated Endometrial Cells

The expression of JARID2 in the peritoneal fluid treated endometrial cells was also examined. While both the control and endo PF treated cells showed an increase in mRNA expression of *JARID2* when compared to media alone, neither was shown to be significant **(Figure 9A)**. Analysis of protein expression of JARID2 showed a significant upregulation of expression when cells were treated with endo PF of 3.61-fold (p=0.0027) and by about 2-fold compared to control PF (p=0.0096) treated cells **(Figure 9B, C)**.

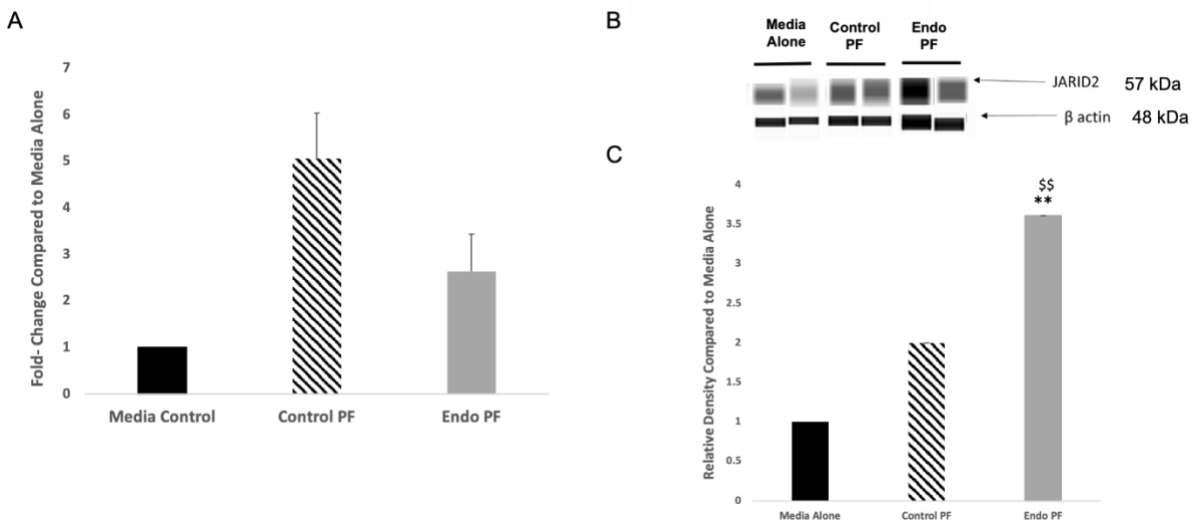


Figure 9: mRNA and protein expression of JARID2 in PF treated endometrial cells. **(A)** mRNA expression of JARID2 in cells treated with control PF (n=12) and endo PF (n=13), relative to expression in a media control (n=6) (p>0.05). (**B)** Representative WES images and densitometric analysis for JARID2 in PF-treated cells. (**C)** Relative protein expression of JARID2 in PF-treated cells was calculated in relation to a media control and presented as a ratio in which media alone is 1. Significant upregulation of JARID2 of 3.61-fold was seen in the endo

PF treated cells when compared to media alone cells (p=0.0027) and by about 2-fold compared to control PF (p=0.0096) treated cells. ** Significant difference (p<0.01) when compared to media alone. $^{\$\$}$Significant difference ($p$<0.01) in mean compared to control PF. Densities of the protein bands obtained were normalized to β-actin or H3. It is to be noted that molecular weights of protein bands in automated WES system differ from the traditional Western blotting, due to differences in technology.

Next, it was assessed if the addition of the PF to the endometrial cells changed the expression levels of the miRNAs that regulate JARID2 levels. miR-148a and miR-29a showed a decrease in expression in both control and endo PF treated endometrial cells, while miR-29a showed a decrease in expression for the control PF treated cells but a slight increase in the endo PF treated cells. Surprisingly, but consistent with what was observed earlier in the EcE tissues, miR-155 showed an increase in expression in both control and endo PF treated cells, but no results were shown to be statistically significant **(Figure 10).** This increase in miR-155 might have lowered the JARID2 expression.

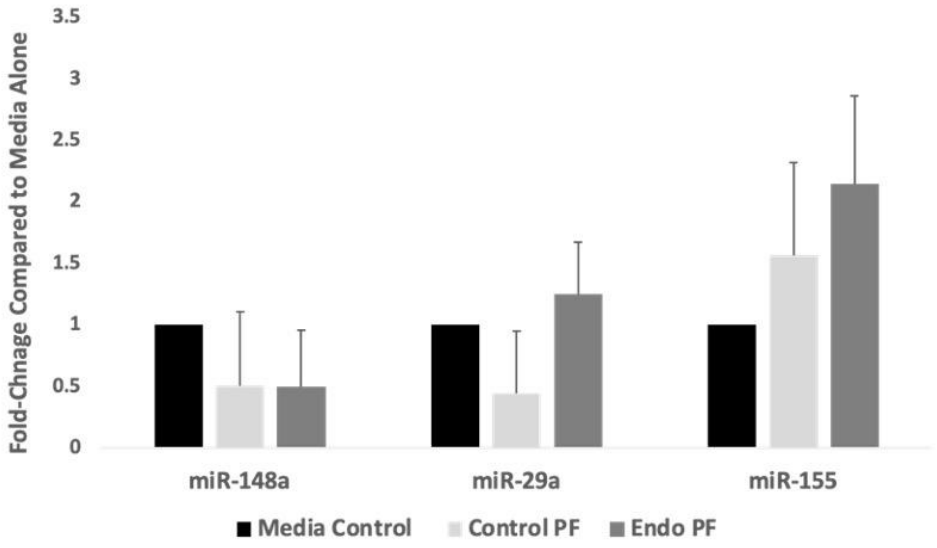


Figure 10: Expression of miRNAs that target JARID2 in PF treated endometrial cells. Compared to media control cells (*n*=4), expression of miR-148a was shown to be lower in the endo treated cells while miR-155 and miR-29a were increased in expression but none were significant (control (*n*=12), endo PF (*n*=13).

FOXP3 mRNA and Protein Expression in Endometrial Tissues and PF Treated Endometrial Cells

With the knowledge that FOXP3 is a regulator of miR-155, the expression levels of FOXP3 in the endometrial tissues and in the PF treated endometrial cells were determined. qPCR showed that while the tissues from patients with endometriosis (EuE and EcE) were slightly upregulated compared to EuN, there was no significance between the expressions **(Figure 11A)**. Relative protein expressions of FOXP3 are shown in **Figure 11B**. No significant difference was seen between the mean density of endo tissue and control tissue bands.

FOXP3 mRNA expression in the endo PF treated cells were shown to be increased in expression but was not significant **(Figure 11C)**. For protein expression using the automated Western blotting system, WES, FOXP3 was increased in cells treated with both control and endo PF, but a statistically significant change in expression was only shown with endo PF treatment (2.32-fold, p=0.0493) **(Figure 11D).**

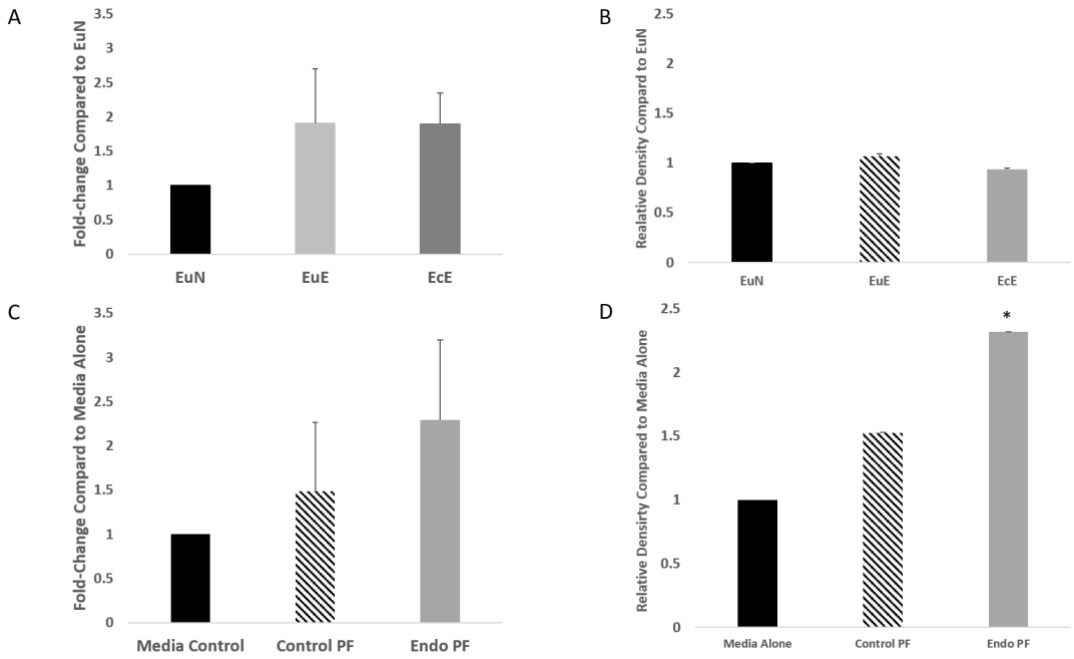


Figure 11: mRNA and protein expression of FOXP3 in patient tissues and PF treated cells

(A) Relative mRNA expression of *FOXP3* in EuN (*n*=5), EuE (*n*=10), and EcE (*n*=6). Upregulation of the FOXP3 mRNA expression in the endometriotic patient tissues was observed but was not significant; **(B)** Relative protein expression of FOXP3 in EuE and EcE was calculated in relation to EuN. Down-regulation of EuE and EcE was seen when compared to EuN. For all comparisons made, $p>0.05$. **(C)** mRNA expression of *FOXP3* in cells treated with control PF (*n*=12) and endo PF (*n*=13), relative to expression in a media control ($p>0.05$). **(D)** Relative protein expression of FOXP3 in PF-treated cells was calculated in relation to a media control and presented as a ratio, in which media alone or EuN is 1. FOXP3 expression was 2.32-fold higher in endo PF ($p=0.0493$) than in control media alone treated cells. *$p<0.05$. Densities of the protein bands obtained were normalized to β-actin or H3.

miR-155 Regulates PRC2 Complex and FOXP3

Since JARID2 and FOXP3 are targets of miR-155, and miR-155 was upregulated in endometriotic tissues and PF treated cells, it was investigated if modulating miR-155 levels using a mimic or inhibitor will alter these target genes. To test this, the expression of JARID2, PRC2 complex, and FOXP3 were determined in endometrial cells transfected with a miR-155 mimic or inhibitor (antagonist). Transfection efficiency of miR-155 is shown in **Figure 12A**. In cells transfected with the mimic, treatment with control PF increased the expression of miR-155 by over 3-fold, compared to when treated with the inhibitor, where the expression decreased below 0.50-fold. In contrast, in cells transfected with the mimic, treatment with endo PF increased miR-155 expression by 1.5-fold but decreased below 0.50-fold after treatment in cells transfected with the inhibitor. Upon PF treatment of the cells transfected with the miR-155 mimic, there was minimal effect on *JARID2* mRNA expression ($p>0.05$), but an increase in *FOXP3* expression in cells treated with control PF was noted. This increase in expression was noted even more so in the endo PF treatments. In contrast, PF treatment of miR-155 inhibitor transfected cells had no major effect on *JARID2* or *FOXP3* mRNA expression **(Figure 12B and C)**.

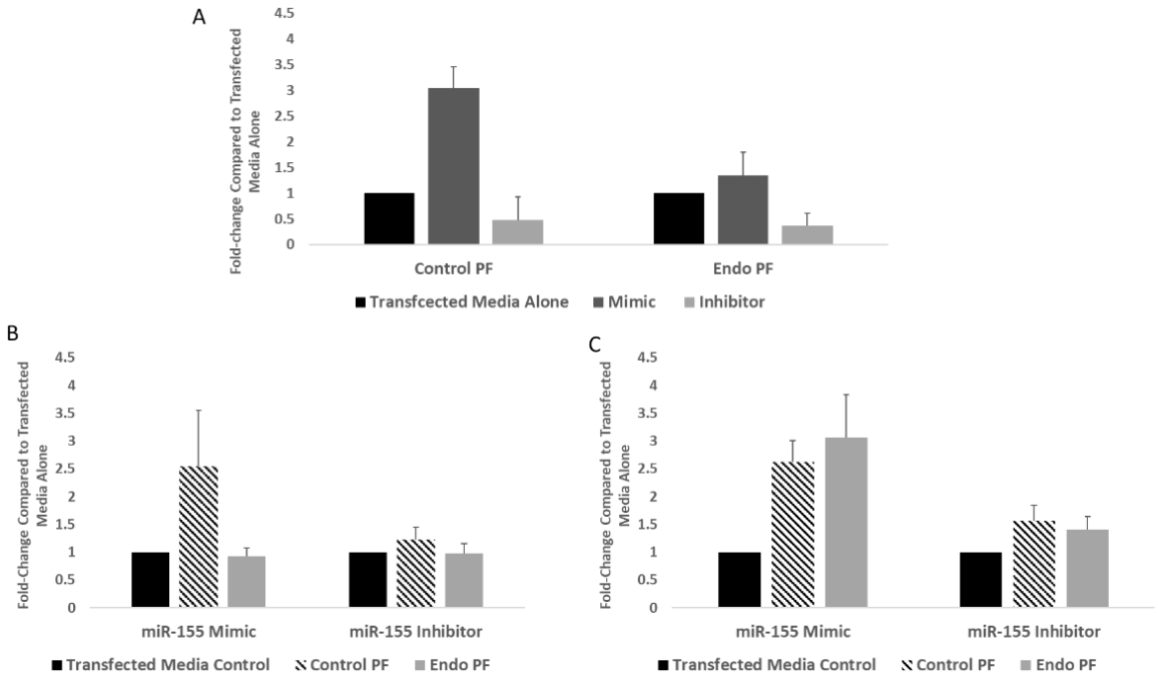


Figure 12: Key mRNA levels in cells transfected with a miR-155 mimic and inhibitor. **(A)**. Levels of miR-155 showing transfection efficiency. **(B)** Transfection with an miR-155 mimic had little effect on *JARID2* expression in PF-treated cells ($p>0.05$), **(C)** but seemed to increase *FOXP3* expression in cells treated with control PF. Compared to control media, the miR-155 inhibitor had no major effect on *JARID2* or *FOXP3* expression in PF treated cells.

Western blotting analysis showed that overexpression of miR-155 resulted in significantly lower JARID2 protein expression in control PF-treated cells compared to endo PF-treated cells ($p=0.0106$). Neither EZH2 nor H2K27me3 protein expression showed any significant up or downregulation in cells overexpressing miR-155 **(Figure 13A)**. In contrast, while both JARID2 and EZH2 showed an upregulation in protein expression in the endo PF treatment groups, when miR-155 was inhibited, no significance was achieved. The protein expression of H3K27me3 in the control PF-treated cells in miR-155 inhibited cells, was

significantly upregulated when compared to both the transfected media alone cells and the endo PF-treated cells p=0.0105 and 0.0138, respectively) **(Figure 13B)**. In miR-155 overexpressing cells, both control and endo PF significantly increased FOXP3 protein expression when compared to transfected media alone (p=0.0005 and 0.0079, respectively). In contrast, no significant difference in FOXP3 protein expression was seen in cells transfected with an miR-155 inhibitor **(Figure 13C)**.

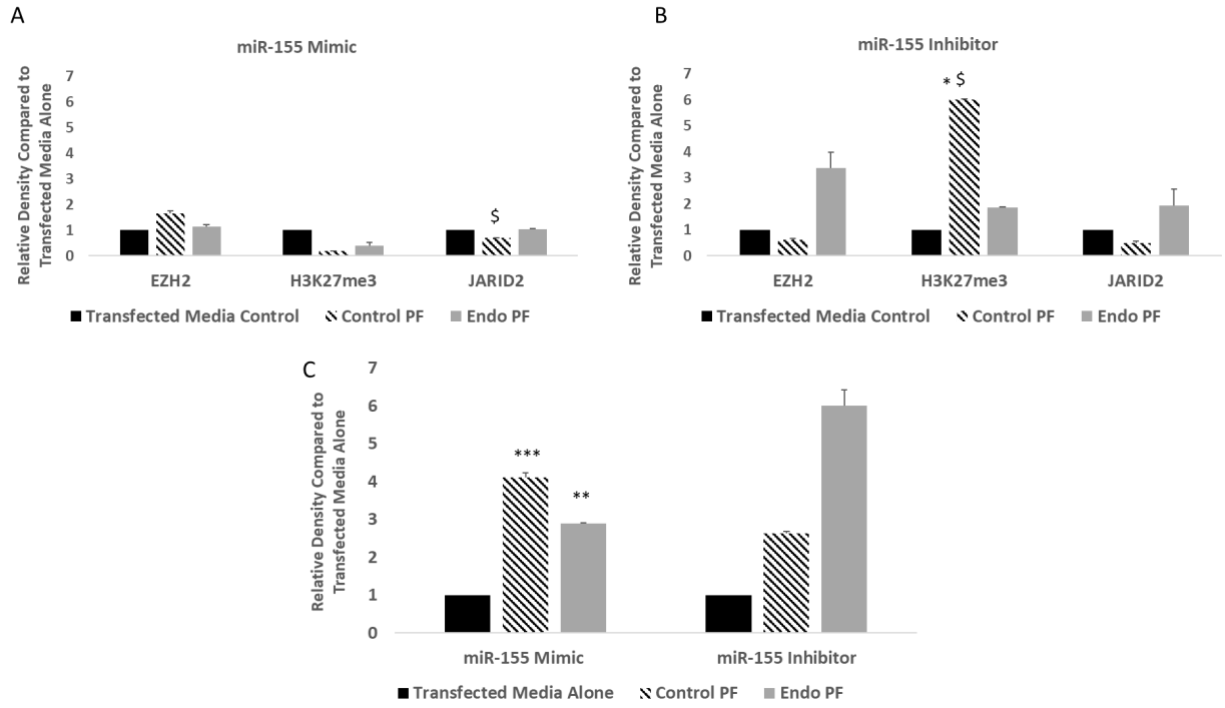


Figure 13: Expression of key targets in cells transfected with miR-155 mimic and inhibitor

A) Transfection with a miR-155 mimic resulted in significantly lower JARID2 expression in control PF-treated cells (n=6) compared to endo PF-treated cells (n=6) (p=0.0106). No significant difference in expression was seen in EZH2 or H3K27me3. **B)** Transfection with a miR-155 inhibitor resulted in higher H3K27me3 for the control PF-treated cells when compared to transfected media and endo PF-treated cells (p=0.0105, 0.0138). Relative expression of EZH2 and JARID2 expression showed no significant increase or decrease in expression in any of the

treated groups. **C)** Transfection with an miR-155 mimic showed significant upregulation of FOXP3 in both control and endo PF-treated cells (p=0.0005, 0.0079) when compared to transfected media alone. Though FOXP3 was induced by 3 or 6-fold in control or endo PF treated cells transfected with miR-155 inhibitor, no significance was observed. * p<0.05, ** p<0.01, and *** p<0.005 compared to transfected media alone, $^{\$}$Significant difference ($p$<0.05) in mean compared to endo PF. Density of protein bands obtained was normalized to β-actin or H3.

ChIP Using JARID2 or EZH2 Antibody Reveals Other Co-Factors of PRC2 Complex

In order to delineate the alterations in the binding partners of EZH2 in the PF treated cells, ChIP was performed using either JARID2 or EZH2 antibodies followed by ChiP-qPCR promoter array of genes associated with polycomb and trithorax complexes in cells treated with or without PF. **Table 2A** provides a list of the focused gene panel involved in polycomb and trithorax complex activity that were differentially expressed in endo PF treated cells when compared to control PF after IP by either JARID2 or EZH2 antibodies. The enrichment of EZH2 after JARID2 IP was lower in endo PF-treated cells compared to control cells. However, EZH1, a polycomb enzyme which is responsible for mono-, di-, or tri-methylation of H3K27, showed an enrichment after JARID2 IP in endo PF treated cells when compared to control PF treated cells but this enrichment was not significant. In contrast, the enrichment of JARID2 after EZH2 IP was over 5-fold higher in endo PF treated cells compared to control PF treated cells. ARID1A, a subunit of the SWI/SNF complex, with an antagonistic relationship with EZH2 [187], showed enrichment after both JARID2 IP and even higher after EZH2 IP.

When comparing the genes involved in polycomb and trithorax complex activity, pulled down by the two antibodies, **(Table 2B)**, JARID2 IP compared to EZH2 IP showed upregulation of 4 genes with *p*-values< 0.05 in control PF treated cells. While no significant *p*-values were seen for any genes in the endo PF treated cells, when JARID2/EZH2 ratio was calculated, all but 7 genes were shown to be downregulated in these cells suggesting that ChIP by EZH2 in endo PF treated cells has more of an effect on the pull-down expression of these genes compared to JARID2. Any *p*-values>0.05 may be due in part to the smaller sample size tested.

A		**JARID2**		**EZH2**	
Symbol	**Gene Name**	**Fold Change (endo PF/ control PF)**	***p*-value**	**Fold Change (endo PF/ control PF)**	***p*-value**
ARID1A	AT rich interactive domain 1A (SWI-like)	3.39	***0.0117***	10.65	0.1342
ASXL2	Additional sex combs like 2 (Drosophila)	4.51	0.2999	8.88	***0.0395***
CXXC1	CXXC finger protein 1	3.70	***0.0139***	8.56	0.1014
DNMT3B	DNA (cytosine-5-) methyltransferase 3 beta	1.13	0.8213	4.25	***0.0165***
EZH1	Enhancer of zeste homolog 1 (Drosophila)	1.4713	0.7986	12.11	0.259
EZH2	Enhancer of zeste homolog 2 (Drosophila)	0.3789	0.5621	12.79	0.2746
INO80	INO80 homolog (S. cerevisiae)	1.50	0.6214	3.76	***0.0182***
JARID2	Jumonji, AT rich interactive domain 2	2.92	0.2653	5.05	***0.0498***
PHF1	PHD finger protein 1	4.55	***0.0316***	13.31	0.1494
PHF19	PHD finger protein 19	0.2890	0.2152	4.76	0.1622

B		**Control PF**		**Endo PF**	
Symbol	**Gene Name**	**Fold Change (JARID2/EZH 2)**	***p*-value**	**Fold Change (JARID2/EZH2)**	***p*-value**
DNMT3B	DNA (cytosine-5-) methyltransferase 3 beta	2.49	***0.0164***	0.66	0.385
E2F6	E2F transcription factor 6	4.52	***0.00027***	0.51	0.4839
EZH1	Enhancer of zeste homolog 1 (Drosophila)	0.91	0.9143	0.34	0.3762
EZH2	Enhancer of zeste homolog 2 (Drosophila)	3.59	0.3551	0.68	0.6713
JARID2	Jumonji, AT rich interactive domain 2	1.6	0.6838	0.92	0.8731
MTF2	Metal response element binding transcription factor 2	15.63	***0.0234***	0.67	0.6671
PHF19	PHD finger protein 19	1.96	0.3848	0.12	0.1440
SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	8.13	***0.039***	0.73	0.7004

Table 2: ChIP-qPCR of PRC2 complex proteins in PF-treated cells. Chromatin Immunoprecipitation (ChIP) was used to analyze interactions between JARID2 and EZH2 and genes associated with the polycomb and trithorax complexes, normalized to IgG. **A.** Fold change

values represent the ratio of enrichment/binding of JARID2 or EZH2 to various genes in endo PF-treated cells (n=3) to enrichment in control PF treated cells (n=3). Genes with a p-value<0.05 are shown as bold and italicized. EZH1 and EZH2 are also shown but did not have a significant p-value for either JARID2 or EZH2 when comparing the two cell treatments. **B.** Fold change values representing the ratio of enrichment/binding in EZH2 precipitated cells (n=3) to enrichment in JARID2 precipitated cells (n=3) for both cell treatments. p-values<0.05 are shown as bold and italicized along with EZH1, EZH2, and JARID2 which did not show significant p-values for either treatment.

PHF19, a Key Co-Factor in the miR-155-JARID2-EZH2 Crosstalk in Endometriosis

The polycomb-like proteins, PHF1 and PHF19, are critical components of PRC2 complex, both of which showed enrichment after EZH2 IP in the endo PF treated cells (fold change, 13.31 and 4.76-fold, respectively). Both these proteins are shown to work with the PRC2 complex in a manner similar to JARID2 in which they form subcomplexes with PRC2 core components and modulate the enzymatic activity of PRC2 and its recruitment [188,189]. Interestingly, PHF19 is also shown to interact with miR-155 to bring the PRC2 complex to its target [190]. In order to validate the ChIP findings, mRNA expression of *PHF19* was determined in the PF treated endometrial cells using qPCR. mRNA analysis revealed that *PHF19* was upregulated in endometrial cells treated with both control and endo PF when compared to media alone cells with the expression of *PHF19* reaching almost 11.5-fold in the endo PF treated cells (p= 0.0474), suggesting that PHF19 may be working along with miR-155 to bring the PRC2 complex to its target and promote endometriosis **(Figure 14)**.

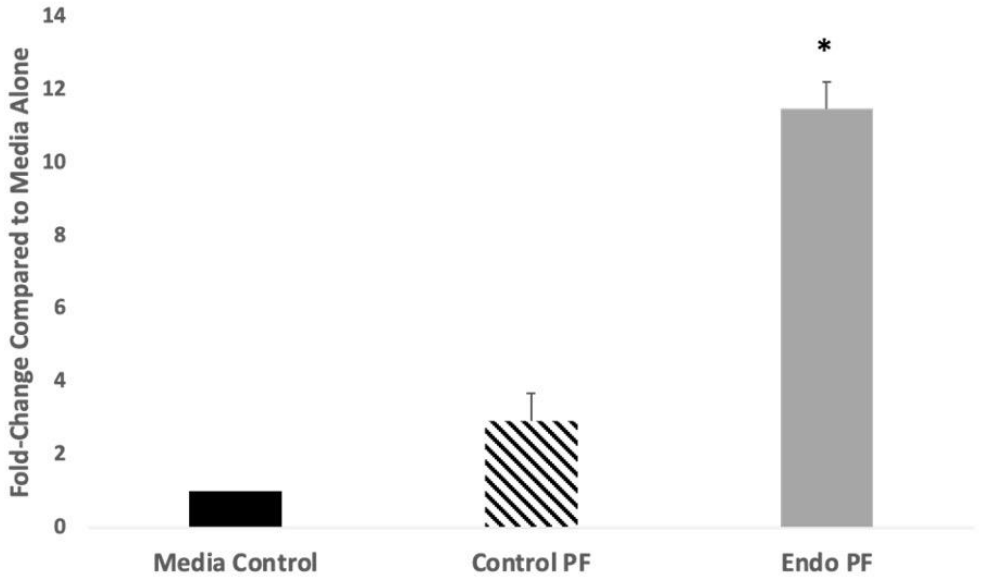
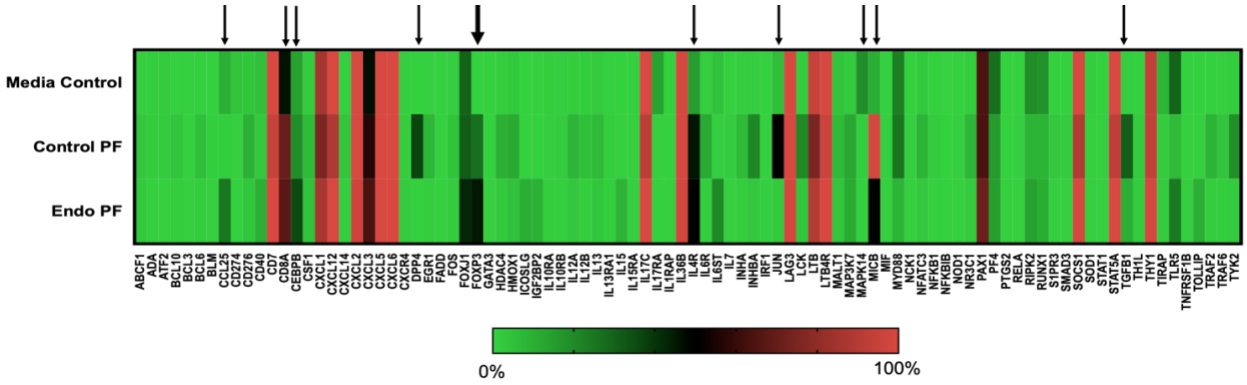


Figure 14: mRNA expression of PHF19 in endometrial cells treated with PF. When Ishikawa endometrial cells were treated with endo PF, expression of *PHF19* was shown to increase 11.50-fold relative to media alone treated cells (p=0.0474). *p<0.05 compared to media alone.

Promoter Methylation of Inflammatory Genes

To assess changes in promoter methylation patterns in PF treated cells, a global DNA methylation array of genes involved in inflammation and autoimmunity was performed. The heat map in **Figure 15A** presents a range (from 0 to 100) of "M", the fraction of input genomic DNA containing 2+ methylated CpG sites in the targeted region of a gene. Genes that were shown to be impacted by DNA methylation by having significant p-values (<0.05) **(Figure 15B)** were C-C Motif Chemokine Ligand 25 (CCL25), Cluster of Differentiation 8a (CD8A), CCAAT Enhancer Binding Protein Beta (CEBPB), Dipeptidyl peptidase 4 (DPP4), forkhead box P3 (FOXP3), interleukin-4 receptor (IL4R), Jun Proto-Oncogene, AP-1 Transcription Factor Subunit (JUN), Mitogen-activated protein kinase 14 (MAPK14), MHC class I polypeptide-related sequence B (MICB), and transforming growth factor beta 1 (TGFB1). All genes had an increased

methylation pattern in cells treated with endo PF compared to the media control. The exception was MAPK14, which decreased in methylation in the endo PF treated cells. FOXP3 M values were 54.02% in endo PF treated cells (p<0.0001), 26.54% in control PF treated cells (p=0.0151), and 0.23% in media control. Bisulfite sequencing will be used in the future to better understand the methylation patterns of sample DNA.



Gene Name	Media vs. Control	Media vs. Endo	Control vs. Endo
CCL25	0.6807	0.2104	**0.0222**
CD8A	**0.0297**	**0.0136**	0.9137
CEBPB	0.8816	**0.0448**	0.1575
DPP4	**0.0005**	0.9996	**<0.0001**
FOXP3	**0.0151**	**<0.0001**	0.0806
IL4R	0.0934	**0.0170**	0.9395
JUN	**0.0006**	0.9048	**<0.0001**
MAPK14	0.1639	**0.0403**	0.9970
MICB	**<0.0001**	**0.0043**	**0.0001**
TGFB1	**0.0052**	0.4354	**0.0403**

Figure 15: Promoter Methylation patterns of inflammatory genes in PF-treated cells. **A)** Heat map showing DNA methylation trends in PF-treated cells on promoters of genes associated with autoimmunity and inflammation. Treatment groups with green shades have lower methylation fractions than those with red shades. Arrows indicate where significant p-values were seen in genes which are shown in **B)** *FOXP3*, is of interest, since it is a tumor suppressor gene and known to regulate both miR-155 and EZH2. Endo PF: M = 54.02%, p=<0.0001; Control PF: M = 26.54%, p=0.0151; Control media: M = 0.23%

DISCUSSION

Our laboratory has been studying mechanisms leading to endometriosis and pain experienced by endometriosis patients [16,24,191,192]. This study stemmed from our previous investigations into the miRNA profile of endometriosis tissues and PF treated cells [17]. Nineteen percent of differentially expressed miRNAs in endo tissues targeted JARID2. Despite the global downregulation seen in the micronome of endometriotic tissues [17], miRNAs that targeted JARID2 were highly expressed in the eutopic tissues of endometriosis patients who also experienced pain as a symptom. The overexpression of miR-148a, miR-29a [17], and miR-155 in endo tissues (Figure 6B) seemed to further support this theory. As shown in Figure 6A, there was an increased expression of PRC2 complex proteins such as *EED* (0.0067), as well as a noticeable trend in overexpression of corresponding genes in ectopic tissues from endometriosis patients, particularly in *EZH2*. This correlates with the findings of Colon-Caraballo and colleagues [151,164] and supports the characterization of EZH2 as a contributor to transcriptional repression and progression of the disease.

Although miR-155 was not originally identified based on the micronome array ($p>0.05$), its relationship with JARID2 has recently drawn the attention of researchers in the field of inflammatory diseases [170,171]. miR-155 seems to be a key intermediate that regulates the crosstalk between JARID2 and PRC2 complex. miR-155 also plays a role in inflammation by working with FOXP3 to promote an inflammatory environment, since it has been shown that FOXP3 induces miR-155 expression [174,178]. Hence, miR-155 is a potential therapeutic target. This study explored the role of miR-155 in endometriosis by studying its interactions with the PRC2 complex, JARID2, and FOXP3. Endometrial cells were transfected with a miR-155 mimic or

antagonist and then exposed to endo or control PF treatments. All PRC2 complex proteins examined showed an increase in expression in endo PF treated cells when compared to media alone treated cells. However, the cells transfected with a miR-155 mimic showed a downregulation of PRC2 complex proteins when exposed to either control or endo PF. The effect of gain-or-loss-of function of miR-155 on *JARID2* was interesting. miR-155 mimic transfected cells treated with control PF showed an increase in *JARID2* expression, while endo PF showed no change in expression. When transfected with the miR-155 inhibitor, no statistical difference in expression was seen in cells treated with control or endo PF. These results were unexpected and suggest that the miR-155 regulation of JARID2 is not sufficient to alter its expression. Hence, other transcription factors and/or epigenetic mediators could play a role in its aberrant expression in endometriosis.

FOXP3 showed a significant increase in expression in both control and endo PF treated cells when transfected with a miR-155 mimic (Figure 13C), which paralleled the results seen for mRNA expression (Figure 12C). Such interactions between miR-155 and FOXP3 have been observed earlier. In diffuse B-cell lymphoma (DLBCL), high FOXP3 expression was correlated with a poor prognosis in patients and when miR-155 was silenced in these cells, there was a parallel decrease in FOXP3 levels [193]. In breast cancer, it was found that FOXP3 and miR-155 work together to down-regulate ZEB2, resulting in reduced invasion [178].

Methylation of the FOXP3 promoter could be partly responsible for pain that women with endometriosis may experience based on the trend of increased methylation in cells treated with PF from endo patients, particularly those reporting pain (Supplementary Figure 2). This has been seen in both biliary atresia and prostatitis [194,195]. Bamidele and colleagues looked at the interaction of EZH2 and FOXP3 in inflammatory bowel disease and found that a mutation in

FOXP3 disrupted EZH2 recruitment and its co-repressive function. They also showed that IL-6 voided the FOXP3-EZH2 interaction and that this destabilized interaction may drive the gastrointestinal inflammation [196]. This disruption in interaction may also be true in endometriosis, since we and others have shown that IL-6 is increased in patients with endometriosis [16,197]. While *FOXP3* mRNA expression in endo PF-treated cells trended to be higher than that of cells treated with control PF, there was no statistical significance observed between the two treatments. These results suggest that FOXP3 is working alongside miR-155 to modulate the expression of EZH2.

EZH2 mRNA expression in cells treated with both control and endo PF was higher when compared to media alone cells, but there was no statistical difference seen. However, an upregulation was seen in H3K27me3 in cells treated with endo PF. This is also significant as H3K27me3 is the downstream target of EZH2 and performs the transcriptional repression in cells [198]. The benefit of studying the PRC2 complex proteins in tissues and treated cells gave us the ability to compare short-term (*in vitro)* and long-term (*in vivo*) effects of peritoneal fluid on endometrial cells. This difference is likely to contribute to explaining the disparities in the observed results.

ChIP-qPCR was used to better understand the regulatory roles of JARID2 and EZH2 and their cross-interactions in endometriosis. By observing how it binds to regulatory elements of various genes, a sense of how the mechanisms described above differ between PF from patients with and without endometriosis was gained. The data presented in Table 2 showed that the pull-down expression of JARID2 by EZH2 IP was over 5-fold higher in cells treated with endo PF compared to control PF. It is interesting to note that, while not significant, JARID2 IP has a fold-change greater than 1 for EZH1, while for EZH2 it is less than 1 for endo PF treated cells. This

suggests that the JARID2 interaction with EZH2 may not be as strong as it is with EZH1, which can also methylate H3K27 to contribute to transcriptional repression. Although it is typically associated with active domains, EZH1 can actually achieve repressive results similar to EZH2 via additional histone modifications [199-201]. It is interesting to note that when comparing genes after immunoprecipitations by the two antibodies (JARID2 and EZH2) in the two PF (endo or control PF) treated cells (Table 2B), the endo PF treated cells showed a trend of having a fold-change less than 1 when comparing JARID2 vs EZH2 IP. This suggests that EZH2 in endo PF treated cells is having more of an effect on the expression of all genes in the array (PRC2 complex core, alternate and binding partners) when compared to JARID2 further supporting a role for EZH2 in endometriosis.

One gene that should be noted and that was shown to have higher fold-change post EZH2 IP in endo PF treated cells vs control PF treated cells was PHF19. PHF19 is a gene silencer and co-factor that can bind H3K36me3, which allows it to act as a recruiter for the PCR2 complex [202,203]. PHF19 also promotes tumorigenesis by the enhancement of the deposition of H3K27me3 and when PHF19 is depleted, this led to a loss of H3K27me3 domains [204]. This suggests that another mechanism which may be at play in transcriptional repression involves PHF19. PHF19 has also been deemed to play a role in the switch from proliferative to invasive states in melanoma cells [205]. Thus, there are studies suggesting targeting PHF19 as an alternate strategy to inhibit EZH2 [206]. This study found that *PHF19* mRNA expression was significantly upregulated in cells treated with endo PF (Figure 14). This suggests that miR-155 and PHF19 may be working together to bring the PRC2 complex to its targets in endometriosis. Putting these results together with miR-155 transfection studies, the study suggests that while miR-155 and PHF19

may be the main helper in regulating the PRC2 complex in endometriosis, JARID2 may be taking up the slack when miR-155 is inhibited.

The findings presented here, as summarized in **Figure 16**, provide potential mechanisms that may be at play in endometriosis patients. This study shows that in the presence of endometrial PF, all the components of the PRC2 complex, along with JARID2, FOXP3, and miR-155, are increased in expression when compared to control PF. Gain-or loss-of function of miR-155 showed an effect on PRC2 complex proteins but not on JARID2 levels. This suggested that other epigenetic regulators may be involved. ChiP-qPCR pull-down studies using JARID2 or EZH2 antibodies in PF treated cells showed alterations in epigenetic proteins associated with either of these complexes. In addition to the known binding partners such as EZH1, DNMT3B etc, the expression of PHF19 (a PRC2 complex co-factor) was highly upregulated in EZH2 compared to JARID2 pull-down assay. This finding, in addition to what is known in the literature [190,203,204,206], suggests that in women with endometriosis, FOXP3/miR-155, in conjunction with PHF19, co-localizes with the PRC2 complex to promote its interaction and function with its targets. This leads to the increased H3K27me3 deposition thus modulating gene transcription. In contrast, this complex has a reverse effect on JARID2, thus preventing its association with the PRC2 complex, unless miR-155 is altered. This novel crosstalk among key epigenetic regulators leads to an increase in inflammation and growth of endometriotic lesions. This opens the door for testing newer targets in addition to the EZH2 inhibitors and miRNA mimics/antagonists currently being tested in endometriosis. For example, although histone demethylase inhibitors are thought to be ineffective against JARID2 due to its lack of true demethylase activity, additional investigations into the role of JARID2 in endometriosis could uncover alternate options to therapeutically regulate it, such as dihydroartemisinin which has

been used in prostate cancer [207]. Additionally, the role for PHF19 as the master-regulator of the miR-155-PRC2 complex-JARID2 crosstalk is also a viable candidate for therapy and should be further explored in endometriosis.

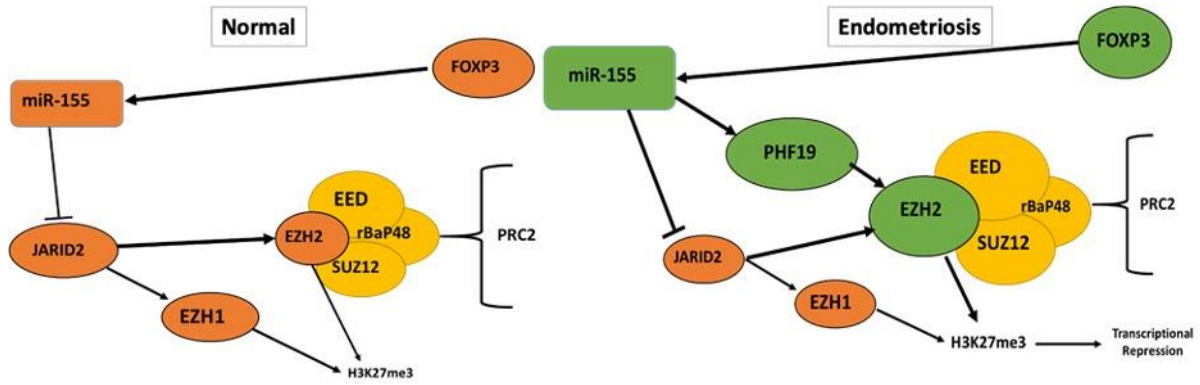


Figure 16: Proposed schematic of the epigenetic crosstalk playing a role in endometriosis. Mechanism proposed in normal women vs women with endometriosis. Arrows indicate activation or general targeting while "T" bars indicate inhibition.

MATERIALS AND METHODS

Human Subject Participants

Women ages 18 to 60 years, undergoing tubal ligation or having non-endometriosis disorders (controls, *n*=12) or patients with endometriosis ("endo", laparoscopically diagnosed followed by pathological confirmation and/or patients with symptoms, *n*=14) were recruited from the Obstetrics-Gynecology clinic at Cabell Huntington Hospital, Joan C Edwards School of Medicine, Marshall University, in Huntington, WV. This HIPAA compliant study was approved by the Institutional Review Board of the Marshall University School of Medicine and was carried out per the principles of the Declaration of Helsinki. All patients were consented prior to the study. The inclusion criteria included women ages 18-60 years old, with normal menstrual cycles and otherwise in normal health (except for pain and endometriosis) who have not been on

any hormonal medication for at least one month before sample collection. Exclusion criteria included subjects with current medical illnesses such as diabetes, cardiovascular disease, hyperlipidemia, hypertension, systemic lupus erythematosis or rheumatologic disease, positive HIV/AIDS, active infection. Subjects were asked to stop multivitamins that contain high levels of antioxidants and anti-inflammatory medications prior to sample collection.

In this study, the majority of the samples were from endo patients diagnosed with stage I/II and only one at stage IV. Pathological confirmation for endo patients classified the patients as mostly belonging to the peritoneal or uterine serosa pathology. All women completed a gynecologic/infertility history form, a pre-operative quality of life questionnaire, and assessment of pain using a visual analog scale for assessment of endometriosis associated pain (dysmenorrhea, non-menstrual pelvic pain, dyspareunia, and dyschesia) (adapted from the validated International Pelvic Pain Society's Pelvic Assessment Form). Date of their last menstrual period was used to assess their cycle time.

RNA and Protein Isolation in Peritoneal Fluid-Treated Cells

Peritoneal fluid (PF) (devoid of blood contamination) was collected on ice from all women during laparoscopic surgery. Peritoneal fluid was spun at 2000*xg* to remove any cellular debris. The supernatant was used immediately for studies or stored in a -80^{0}C freezer for future use. To establish a cell model of the peritoneal environment, Ishikawa cells, a human (39-year-old woman) established endometrial adenocarcinoma epithelial cell line (Cat No: 99040201, Sigma-Aldrich, St. Louis, MO), were cultured in T75 flasks in complete media (DMEM/F12, 10% FBS, 1% Pen/Strep, 1% L-glutamine). These cells were used because they express characteristics similar to those of mature endometrial epithelial cells [208-210]. Approximately 70% confluent cells were treated with 1% PF from patients for 48 hours in a DMEM/F12 media

containing 1% charcoal-stripped FBS. Patient peritoneal fluid (PF) groups were control PF (fluid from women without endometriosis) and endo PF (fluid from women with endometriosis). The concentrations of PF chosen were based on our previous published studies [16,17]. At the end of the 48-hour treatment, cells were collected using Qiazol Lysis reagent (Cat No: 79306, Qiagen, Gaithersburg, MD) and RNA was isolated using the Qiagen miRNeasy Mini Kit (Cat No: 217004, Qiagen, Gaithersburg, MD). The quantity and quality of RNA were measured in the NanoDrop 2000 spectrophotometer. Cell lysates for measuring proteins were prepared in RIPA buffer containing protease inhibitors (Cat No: P2714, Sigma-Aldrich, St. Louis, MO), and protein concentrations were measured using a modified Lowry protocol [211].

Endometrial Tissue Collection and RNA/Protein Isolation

Endometrial (eutopic) tissues from control patients (EuN), eutopic tissues from endometriosis (peritoneal endometriosis, "endo") patients (EuE), and ectopic endometriotic tissues (EcE) from endo patients were removed during laparoscopy/laparotomy by a qualified physician. Biopsy fragments were immediately placed in RNA*later* solution (Cat No: 76104, Qiagen, Gaithersburg, MD) and subsequently stored in a freezer at -80°C. RNA extraction from 100 mg of tissue (eutopic and ectopic) was carried out using Qiazol Lysis Reagent (Cat No: 79306, Qiagen, Gaithersburg, MD). Tissues were homogenized using zirconium oxide beads in a Bullet Blender® homogenizer (SKU: BBX24, Next Advance, Troy, NY) and RNA was isolated using the Qiagen miRNeasy Mini Kit following the manufacturer's recommendations (Cat No: 217004, Qiagen, Gaithersburg, MD). The quantity and quality of RNA were measured using the NanoDrop 2000 spectrophotometer (Cat No: ND2000, Thermo Scientific, Waltham, MA). Protein lysates from 50 mg of tissue were homogenized in RIPA buffer prior to protein estimation by a modified Lowry method [211].

mRNA and miRNA Expression in Tissues and PF-Treated Endometrial Cells

RNA (which includes miRNA) isolated from the tissues and treated cells was used. cDNA synthesis from 1 µg of each sample was performed using iScript cDNA synthesis kit (Cat No: 1708890, Biorad, Hercules, CA). mRNA expression was analyzed in the cDNA samples using SYBR Green (Cat No: 1725270, Biorad, Hercules, CA), and the primers listed in **Supplementary Table 1**. 18S was used for normalization of mRNA expression. For determining miRNA expression, cDNA synthesis from 2 µg of each sample was performed using miScript II RT Kit (Cat No: 218161, Qiagen, Gaithersburg, MD). Following cDNA synthesis, the expression of miR-29a, miR-148a, and miR-155 in tissues and PF-treated cells were determined using the appropriate Qiagen Primer Assay Kit, as per the manufacturer's instructions. A primer assay for RNU6 was used as housekeeping for miRNA expression.

Protein expression in PF-treated cells and patient tissues

Total protein was measured using a modified Lowry method. Protein (7µg for cells and 5 µg for tissues) was run on the automated western blotting system, WES [212] (Cat No: 004-600, Protein Simple, San Jose, CA). The primary anti-rabbit antibodies for EZH2 (1:50, Cat No: 5426S), FOXP3 (1:25, Cat No: 12632S), JARID2 (1:50, Cat No: 13594S), and H3K27me3 (1:25, Cat No: 9733S) (Cell Signaling, Danvers, MA), anti-rabbit β-actin (1:100, Cat no: 4970S, Cell Signaling, Danvers, MA), and anti-rabbit H3 (1:100, Cat No: 39451, Active Motif, Carlsbad, CA) were used to measure expression levels within the samples. HRP-conjugated rabbit secondary antibody provided in the WES kit was used. Plates (12-230 kDa and 25 capillary) were run using default settings and results were analyzed using the Compass for WES software (Version 5.0.1). Band area given by the software was used and normalized to β-actin or H3. Results were expressed as a ratio in which media alone (for cell treatments) or EuN (for

patient tissues) was 1. It is important to note that proteins examined using the automated Western Blotting system, WES, will have different expected molecular weights compared to traditional Western blotting due to differences in technology.

Cell Transfection with miR-155 Mimic/Inhibitor

Cells were transfected using SiPORT™ NeoFX ™ transfection agent (Cat No: AM4510, Ambion, Austin, TX) as recommended by the manufacturer. In short, the SiPORT™ NeoFX ™ was diluted in Opti-MEM® Reduced Serum Media (Cat No: 31985062, Invitrogen, Carlsbad, CA) and incubated for 10 min at room temperature. miR-155 mimic (Pre-miR™), inhibitor (Anti-miR™), positive control (anti-let-7c) (Cat no: 4392431, Thermo Scientific, Waltham, MA), and negative control (Negative control #1) (Cat No: AM17010, Thermo Scientific, Waltham, MA) were diluted in cell media (DMEM/F12, 10% FBS, 1% Pen/Strep, 1% L-glutamine) to a final concentration of 30 nM and then combined with the transfection agent and incubated for 10 min at room temperature. Transfection mixtures were added to 6-well plates and overlaid with cell suspensions. Cells were then incubated for 24 hours prior to treatment with peritoneal fluid from control and endometriosis patients, as previously described. Transfection efficiency was tested by collecting cells in Qiazol and assessing miRNA expression using the miR-155 primer assay. RNA was isolated using the miRNeasy Mini Kit following the manufacturer's recommendations (Cat No: 217004, Qiagen, Gaithersburg, MD). RT-qPCR was used (as previously described) to determine the expression of key downstream targets such as JARID2 and FOXP3.

Western blots were performed in the traditional manner. Total protein was measured using a modified Lowry method. Protein (35µg) was separated on a 4-20% Tris-HCl gradient gel (Cat No: 4561096, Biorad, Hercules, CA) and transferred onto nitrocellulose membranes. After

washing with Tris-buffered saline with Tween 20 (TBST), the membranes were blocked in 5% bovine serum albumin or 5% milk in TBST for 1 hour, then incubated at 4°C overnight with anti-rabbit antibody against JARID2 (Cat No: 13594S), FOXP3 (Cat No: 12632S), EZH2 (Cat No; 5426S), and H3K27me3 (Cat No: 9733S) (1:1000, Cell Signaling, Danvers, MA) and anti-mouse against β-actin (1:4000, Cat No: A5316, Sigma-Aldrich, St. Louis, MO). Anti-rabbit antibody against H4/H3 was diluted 1:20000 (Cat No: 07-108, Sigma-Aldrich, St. Louis, MO). Dilutions for primary antibodies vary from those used for WES due to the different methods used. The membranes were washed and incubated with HRP-linked anti-rabbit or anti-mouse secondary antibody (1:6000, Cat No: A6154, A4416, Sigma-Aldrich, St. Louis, MO) for one hour at room temperature. After washing, membranes were developed in HRP Substrate (Cat No: WBKLS05000, Millipore, Temecula, CA) and imaged using the ChemiDoc system (Cat No: 1708265, Biorad, Hercules, CA). Densitometric levels of protein bands were quantified and normalized to β-actin or H3. Results were expressed as a ratio in which media alone was 1.

EpiTect Methylation Array

An EpiTect Methyl II Complete PCR Array (Cat No: 335005, Qiagen, Gaithersburg, MD) was used to examine the levels of methylation in genes involved in inflammation and autoimmunity in PF treated Ishikawa endometrial cells. Cells were treated as previously described and collected. DNA was isolated from the cells and DNA quantity and quality of RNA were measured using the NanoDrop 2000 spectrophotometer (Cat No: ND2000, Thermo Scientific, Waltham, MA). Protocol provided by the manufacturer was followed. Input DNA was obtained and aliquoted into four equal portions and subjected to mock, methylation-sensitive, methylation-dependent, and double restriction endonuclease digestion. After digestion, the enzyme reactions were used for qPCR using Sybrgreen. Analysis was performed using the

algorithm provided by Qiagen/SA Biosciences (Gaithersburg, MA). A heat map was created from the data provided using Prism software (Version 9.0.0) (GraphPad, Inc., La Jolla, CA).

Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) was performed using the Chromatrap ChIP-Seq kit (Cat No: 500189, Porvair, Ashland, VA) using either JARID2 or EZH2 antibodies. Approximately 70% confluent Ishikawa cells were treated with 1% PF from patients for 48 hours in a DMEM/F12 media containing 1% charcoal-stripped FBS. Proteins were cross-linked by adding formaldehyde (0.75% by volume) and allowing for a 10-minute incubation at room temperature. Glycine (0.5M) was added and incubated for an additional 10 minutes. Cells were twice rinsed with PBS, collected in 1 ml PBS and pelleted by centrifugation. All other buffer and components used were obtained from the kit. Protocol v1.5 of the manufacturer's instructions was followed (Porvair, Ashland, VA). Cells were suspended in 800 µL of hypotonic buffer before being centrifuged and the nuclear pellet was separated and resuspended in 400 µL of pre-warmed lysis buffer. Sonication was performed using a Covaris ME220 (SKU: 500506, Woburn, MA). Each sample was aliquoted into 3 different tubes prior to sonication. Each tube was sonicated for 100 seconds and sheering efficiency was verified using an agarose gel as a smear of DNA fragments between 100-500 bp in length. ChIP-grade anti-JARID2 antibody (Cat No: 13594, Cell Signaling, Danvers, MA) and EZH2 (Cat No: 5246S, Cell Signaling, Danvers, MA) was used for antibody precipitation. DNA concentration was determined by NanoDrop 2000 spectrophotometer. The Human Polycomb & Trithorax Complexes EpiTect ChIP qPCR Array (Cat No: 334211 GH-506A, Qiagen, Gaithersburg, MA) consisting of primers for genes belonging to the polycomb and trithorax complexes (core, alternate, and additional components), as well as polycomb co-factors such as PHD finger protein 19 (PHF19) and heterochromatin

(CBX) proteins, was run for all samples. Percent enrichment and further statistical analysis were calculated using algorithm provided by Qiagen/SA Biosciences (Gaithersburg, MA).

Statistical Analysis

Prism software (Version 9.0.0, GraphPad, Inc., La Jolla, CA) was used for analysis of all the non-array qPCR and WES data obtained from human tissue and cell culture studies. All values were expressed as mean ± standard error of the mean (SEM). A one-way ANOVA followed by Tukey's post-hoc test was used to detect any significant p-values. *p* Values less than 0.05 were considered to be significant.

Author Contributions: Conceptualization, S.B., K.R.W, and N.S.; methodology, S.B., K.R.W, and N.S.; software, S.B., K.R.W.; validation, S.B., K.R.W, and N.S.; formal analysis, S.B. and K.R.W.; investigation, S.B., K.R.W, and N.S.; resources, B.M.; data curation, S.B., K.R.W, and N.S.; writing—original draft preparation, S.B. and K.R.W; writing—review and editing, all authors; visualization, S.B., K.R.W, and N.S.; supervision, B.M.; project administration, all authors; funding acquisition, S.B., and N.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Marshall University School of Medicine (Protocol IRBNET3: 114954-25 and date of approval=13 May 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data sharing not applicable

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Conflicts of Interest: The authors declare no conflict of interest

Gene	Sequence
18S	F: GCAATTATTCCCCATGAACG
	R: GGCCTCACTAAACCATCCAA
EED	F: CATTGGGCAATCAAGTTGGCA
	R: ACAAGTGTGGAGAAAAAGCCTG
SUZ12	F: GTTACCGGTGAAGAAGCCGA
	R: TTGGCTTCTCAAAGGCCTGG
EZH2	F: AAGGAGTTTGCTGCTGCTCT
	R: ATTAATGGTGGGGGTGCTGG
JARID2	F: CTGCAGCACAAACGTGACTT
	R: CATCAGCGAAACGTGAAGGTC
FOXP3	F: ACTGGGGTCTTCTCCCTCAA
	R: GGGATTTGGGAAGGTGCAGA
PHF19	F: AATCCGTGGTCCCTATCCCA
	R: ATAGGAGTCCCGAGTCCCTG

Supplementary Table 1: Primer sequences for RT-qPCR analysis of epigenetic gene expression in patient tissues and PF-treated cells. Primers were designed using NCBI GenBank and ordered from Invitrogen.

CHAPTER 3

EPIGENETIC REGULATION OF CXCR4 AXIS IN ENDOMETRIOSIS

To be submitted for publication

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ABSTACT

Many researchers believe that epigenetics plays a significant role in the initiation and progression of the gynecological disease endometriosis. One of the main genes examined in endometriosis is EZH2 and its trimethylation of H3K27. Here, using peritoneal fluid (PF) from IRB-approved and consenting patients with and without endometriosis, we treated endometrial cells, as well as eutopic tissues from patients without endometriosis. Performing RT-qPCR and western blotting to measure expression of EZH2 and H3K27me3, we noted a higher expression of these genes in cells and tissues treated with endometrial PF. The use of a CXCR4 inhibitor, AMD3100, decreased expression of all the genes examined in the PF treated cells and tissues and decreased invasion of the cells, but increased proliferation of the endometrial cells. The use of an EZH2 inhibitor, GSK126, also decreased this expression in both the cells and tissues as well as decreasing the proliferation of the endometrial cells. However, the use of GSK126 significantly increased the expression of the chemokine CXCR4 in endometrial tissues when added in the presence of endo PF and increased the invasion of the cells. A combination treatment using both GSK126 and AMD3100 decreased the expression of all genes in both the treated cells and tissues, and decreased

cell proliferation and invasion. Thus, our studies show dual results for cell proliferation and migration when the inhibitors were added to cells and tissues separately. They also show that a combination approach targeting both CXCR4 and EZH2 may be a more successful treatment for women with endometriosis.

INTRODUCTION

Endometriosis affects 1 in 10 women, though recent studies indicate this ratio may have increased to 1 in 9 women [1]. These numbers equate to about 176 million women worldwide. Some of the major symptoms associated with endometriosis include heavy bleeding and dysmenorrhea, dyspareunia, and infertility [2,213]. Though the pathophysiology of this disease is not completely understood, few theories that have been proposed, [9,11,214], include Sampson's theory, also known as the retrograde menstruation theory, the coelomic metaplasia theory, and the embryonic origin theory. Other recent theories include stem cells, genetics, and autoimmune disease [14,15].

All of the accepted theories in endometriosis support a pathological role for an inflammatory peritoneal microenvironment. This microenvironment differs between women with and without endometriosis, in that the former group, peritoneal fluid (PF) is present in larger quantities and contains excess levels of inflammatory cells, pain-inducing molecules, growth promoters, and cytokines/chemokines. This pelvic inflammatory environment leading to endometriosis has been widely investigated by various groups, including our own [16-19,21-25]. We and others have also shown that oxidants present in abundance in the PF can increase the proliferation and growth of endometrial cells [215].

Endometriosis is also a highly inflammatory disease with many inflammatory markers being highly expressed in the peritoneal fluid of women with endometriosis [216,217]. Lymphokines

have been shown to be present in excess in the PF of women with endometriosis. One function of lymphokines is to stimulate monocytes to enter the peritoneal cavity from the bloodstream, where they can transform into macrophages. These peritoneal macrophages are known to be pro-inflammatory due to their release of growth factors into the peritoneal cavity. Some of these growth factors include tumor necrosis factor α (TNF-α), IL-1, and IL-6 [30]. All of these have been shown to be upregulated and play a role in endometriosis [31,34]. Macrophages have also been studied by our laboratory and have been found to play a role in the progression of endometriosis [21,29,46,48,55,218]. Macrophages have also been known to produce vascular endothelial growth factor (VEGF) and are regulated by IL-10 and are hypoxia dependent [219]. IL-10 has been noted to be high in serum levels of women with endometriosis, promoting the growth of endometrial lesions [220]. VEGF has been shown to be increased in the ectopic endometrium, as well as increased in the PF of women with endometriosis when compared to normal women [44]. Expression of VEGF has also been shown to correlate with expression of CXCR4 [221].

C-X-C Motif Chemokine Receptor 4, CXCR4, is one of the most widely expressed chemokine receptors [222], as well as being an inflammatory pathway that has been of recent interest and exploration in endometriosis. Binding to its only ligand CXCL12, or SDF-1, CXCR4 activates mechanisms that are important in reproductive biology [223] , as well as triggers signaling pathways important in cell migration [222]. CXCR4 has also been shown to bind macrophage inhibitory factor (MIF), a pro-inflammatory cytokine[224]. The binding of CXCR4 and MIF has been shown to increase tumor growth, epithelial-to-mesenchymal (EMT), and the production of IL-6 in non-small cell lung cancer [225]. This may be important in endometriosis as there has been shown to be increased IL-6 concentrations in the PF of women with endometriosis [34]. CXCR7 is another heptahelical G protein-coupled receptor for CXCL12. Patients with deep infiltrating endometriosis

(DIE) showed a higher expression of CXCR4 in eutopic endometrial stromal cells compared to DIE-derived stromal cells and increased concentrations of CXCL12 in the PF. It was suggested that the eutopic endometrial stromal cells that expressed CXCR4 on their membrane were attracted to the peritoneal cavity due to the high concentrations of CXCL12 in the PF [226]. A recent study used a bone marrow transplantation mouse model and showed that CXCR4 and CXCR7 are highly expressed in the bone marrow-derived cells engrafting endometriosis and that when either of those receptors were targeted, lesion size was decreased compared to untreated controls [227].

Targeting CXCR4 has been explored as therapy in other diseases. One such compound, AMD3100, common name Plerixafor, is a highly selective CXCR4 chemokine receptor antagonist with an IC_{50} value of 44 nM in cell-free assay was originally developed as an inhibitor for HIV-1 and HIV-2 replication. This compound inhibits the intracellular calcium signaling and chemotactic response elicited by CXCL12 [228]. In endometriosis, Ruiz, et al. showed that AMD3100 alone decreased migration but increased invasion of the cells, however when AMD3100 was added in presence of the ligand CXCL12 both migration and invasion were decreased [229]. These studies proposed targeting this axis as a potential non-hormonal therapy for endometriosis.

Recently, we published a paper examining the epigenetic mechanisms in endometriosis and the effect of peritoneal fluid (PF) from women with endometriosis affects those mechanisms. Specifically, we examined the effect of PF on the Polycomb repressive complex 2 (PRC2) and its major subunit Enhancer of Zeste Homolog (EZH2) [91]. EZH2 is a histone methyltransferase that plays a key role in transcriptional repression through chromatin remodeling, thus regulating gene expression, maintaining cell identity, and oncogenesis. EZH2 activates H3K27me3 thus silencing a broad range of genes [67-70]. Studies have shown elevated EZH2 expression in various cancers [230-232], thus making it a major drug target in the treatment of cancer [233]. We noted an increased

expression of this complex in both the eutopic and ectopic tissues from women with endometriosis, as well as cells treated with endometriotic PF [91]. An increased expression of EZH2 and H3K27me3 has also been shown by Colon-Caraballo et al. as well as others [88,93]. GSK126 is a selective, S-adenosyl-methionine-competitive small molecule inhibitor of EZH2 methyltransferase activity which is more than 1,000-fold selective for EZH2 over other histone methyltransferases [234]. Taken together, these studies suggest the use of EZH2 inhibitors as potential therapeutics for endometriosis.

Interestingly, a positive correlation between the expression of EZH2 and CXCR4 has also been seen [235,236] as well as H3K27me3 histone modifications being enriched at the CXCR4 promoter in human embryonic stem cells [237]. Keeping these above studies as a premise, we hypothesized that the peritoneal fluid is a dynamic player in the epigenetic regulation of the CXCR4 axis. We studied the ability of peritoneal fluid from women with endometriosis to modulate the CXCR4-CXCL12-CXCR7 axis as well as the effects of blocking these PF mediated effects.

Using endometriotic PF treated endometrial cells and tissues we showed a possible epigenetic regulation of the CXCR4 axis which resulted in altered proliferation or migration of cells. These studies also indicated dual effects of the currently available CXCR4 or EZH2 inhibitor in the absence or presence of endometriotic PF on endometrial cells and tissues. Hence, these results indicate that a combination of drugs inhibiting both CXCR4 and EZH2 might be more effective in endometriosis than the use of these drugs individually.

MATERIALS AND METHODS

Human Subject Participants

Women ages 18 to 60 years, undergoing tubal ligation or having non-endometriosis disorders (controls) or patients with endometriosis ("endo", laparoscopically diagnosed followed by pathological confirmation and/or patients with symptoms) were recruited from the Obstetrics-Gynecology clinic at Cabell Huntington Hospital, Joan C Edwards School of Medicine, Marshall University (Huntington, WV). This HIPAA compliant study was approved by the Institutional Review Board of the Marshall University School of Medicine and was carried out per the principles of the Declaration of Helsinki. All patients were consented prior to the study. The inclusion criteria included women ages 18-60 years old, with normal menstrual cycles and otherwise in normal health (except for pain and endometriosis) who have not been on any hormonal medication for at least one month before sample collection. Exclusion criteria included subjects with current medical illnesses such as diabetes, cardiovascular disease, hyperlipidemia, hypertension, systemic lupus erythematosis or rheumatologic disease, positive HIV/AIDS, active infection. Subjects were asked to stop multivitamins that contain high levels of antioxidants and anti-inflammatory medications prior to sample collection.

Patients for this study were between the ages of 23-49 years. All women completed a gynecologic/infertility history form, a pre-operative quality of life questionnaire and assessment of pain using a visual analog scale for assessment of endometriosis associated pain (dysmenorrhea, non-menstrual pelvic pain, dyspareunia, and dyschesia) (adapted from the validated International Pelvic Pain Society's Pelvic Assessment Form). Date of their last menstrual period was used to assess their cycle time.

Of the 17 different endometrial PF samples that we used for this study, 8 were stage I/II and 4 were stage IV. Pathological confirmation for endo patients revealed that most of the patients belonged to the peritoneal, uterine serosa, or ovarian pathology. Only 3 women with endometriosis and whose samples we obtained indicated that they did not experience pain symptoms. Peritoneal fluid (PF) (devoid of blood contamination) was collected on ice from all women during laparoscopic surgery. Peritoneal fluid was spun at 2000 x *g* to remove any cellular debris. The supernatant was used immediately for studies or stored in a -80ºC freezer for future use. Twelve different control PF samples were used in this study, with 5 of those indicating that they did experience pain. All patients were undergoing laparoscopic hysterectomy or tubal ligation. 2 patients were noted to have an adhesion or fibroid present, while three others were noted for uterine serosa or adenomyosis pathology but were not shown to be positive for endometriosis.

The eutopic endometriotic tissue was obtained from women without endometriosis undergoing a laparoscopic hysterectomy. The excised tissues were either used immediately or placed in a -80º freezer for future use.

PF Treatment of Endometrial Stromal Cells and Eutopic Tissues

Primary stromal endometrial cells, EOOF5 (a gift from Emory University isolated from eutopic endometrium obtained from a 34-year-old woman without endometriosis undergoing surgery for tubal ligation), were cultured in T75 flasks in complete media (DMEM/F12, 10% FBS, 1% Pen/Strep, 1% glutamine). Cells were stained with vimentin to confirm that were of stomal origin. When cells were about 80% confluent, media was changed to a DMEM/F12 media containing 1% charcoal-stripped FBS (1% CS-FBS media) before being treated with either 1% PF

diluted in cell culture media from women with (endo PF) or without (control PF) endometriosis alone for 48 hours, 1 or 2.5 μM GSK126 (CAS No: 1346574-57-9, Cayman Chemical, Ann Arbor, MI), 1 μM AMD3100 (CAS: 155148-31-5, Sigma-Aldrich, St. Louis, MO), or 1 μM AMD3100+1 μM GSK126 alone for 48 hours, or 1% control or endo PF for 24 hours before adding 1 or 2.5 μM GSK126, 1 μM AMD3100, or 1 μM AMD3100+1 μM GSK126 for an additional 48 hours. Cells were collected for protein and RNA analysis at the end of the time points. EOOF5 cells treated with 1% CS-FBS media alone were used as the control group for data comparison. Concentration of AMD3100 were taken from studies performed by Mishra et al. as well as an MTT assay from our laboratory (data not shown) [238]. Concentrations of GSK126 used on the cells were taken from a study performed by Oki et al. as well as MTT assays performed by our own laboratory (data not shown) [239].

Eutopic endometrial tissue was washed twice using Hanks' Balanced Salt Solution (HBSS, Cat No: SH 30268.02, HyClone, Marlborough, MA) prior to use. 50 mg aliquots of tissue were placed into each well of a 24 well plate. 500 μL of media (DMEM/F12, 10% FBS, 1% Pen/Step, 1% Glutamine) was added to each well and then placed in the 37°C incubator for 4 hours. After 4 hours, tissues were treated with 1% control or endo PF alone, 1,3, 5 or 9 μM GSK126 alone (CAS No: 1346574-57-9, Cayman Chemical, Ann Arbor, MI), or 1% PF plus 1, 3, 5 or 9 μM GSK126. Another subset of tissues was treated with 1 or 2.5 μM AMD3100 (CAS: 155148-31-5, Sigma-Aldrich, St. Louis, MO) or 1 μM AMD3100+ 1 μM GSK126 alone or in combination with 1% PF. For PF or drug(s) alone treatments, the compounds were added for 48 hours before collection. For combination treatments, PF was added to the tissues for 48 hours before drug(s) was added for an additional 48 hours and then collected. 5 different tissue samples were used (3 fresh and 2 frozen) and n=6 control and endo PF samples were used for treatments. All treatments were performed in

duplicates, one was used for protein analysis and the other for RNA isolation. Tissue treated with 1% CS-FBS media alone was used as control for comparison. Various concentrations of AMD3100 and GSK126 were used based on cell results along with providing additional concentrations to determine effects within the tissues.

Cell Proliferation using xCELLigence

EOOF5 cells were used to test cell proliferation under various conditions using xCELLigence technology (Cat No: 05469759001, Agilent, Santa Clara, CA). This technology uses modified 16-well plates (E-plates, Cat No: 5469813001, Agilent, Santa Clara, CA) in which microelectrodes are attached at the bottom of the wells in which cell impedance or cell index (CI) can be measured. In short, the more cells that are adhered, the higher the CI measurement. 100 μL of complete media was added to each well and the background read prior to the plating of the cells. Media was removed from all wells except a media alone well after the reading. 5,000 cells per well were plated in 100 μL of complete media and placed back on the xCELLigence machine for 24 hours and readings were taken every hour. After 24 hours, media was removed from all wells and 1% CS-FBS media was added before beginning various cell treatments. Cell treatments included 1% control or endo PF alone (n=8 for control and n=7 for endo), 1 or 2.5 μM GSK126 or AMD3100 alone, 1% PF plus 1 or 2.5 μM GSK126 or AMD3100, or a combination of 1 μM AMD3100 and 1 μM GSK126 alone and in the presence of 1% PF. Both compounds were added at the same time for the combination treatments. Once all treatments were added to appropriate wells, the plates were left in the xCELLigence machine for 100 hours and continuous readings were taken every hour. All treatments were performed in triplicates and the CI averages were compared to 1% CS-FBS media only wells as a percentage of cell growth.

Scratch or Wound Healing Assay

A scratch assay was used to see how the various treatments could affect the wound healing ability of the EOOF5 cells. 24-well plates were used in which 15,000 cells in 500 μL of complete media were added per well and allowed to grow overnight in the incubator. The next day, media was vacuumed off and a scratch was made down the middle of the well and then washed with Hanks' Balanced Salt solution (HBSS, Cat No: SH 30268.02, HyClone, Marlborough, MA) to remove any debris. 1% CS-FBS was added to each well and images were taken. Various treatments were performed on the cells after initial images were obtained. Treatments were the same as the cell proliferation studies. Plates were placed back in the incubator and images taken every 24 hours for 96 hours. The rate of wound healing (filling of the gaps made by the scratch by movement of cells) for each treatment was compared to the rate of wound healing for the 1% CS-FBS wells, as well as each treatment to other treatments to determine if the rate was faster or slower than another.

Cell Migration using xCELLigence

xCELLigence technology (Cat No: 05469759001,_Agilent, Santa Clara, CA was used to examine how the migration potential of EOOF5 cells could possibly change under various conditions. We used 16-well CIM16 plates (Cat No: 5665825001, Agilent, Santa Clara, CA) in which two different chambers are present. Electrodes attached to the bottom of the wells of the bottom chamber measure the number of cells that had passed from the top chamber to the bottom (migrated) providing a cell impedance (CI) number. First, 160 μL of 1% CS-FBS media was added to each well of the bottom chamber making sure a meniscus has formed. The top chamber was placed on the bottom chamber before adding 30 μL of 1% CS-FBS media to each well. Plates were placed in the incubator for one hour before a background scan was taken. 20,000 cells per well were added to the top plate along with various cell treatments, which were the same as in our cell

proliferation and scratch assay studies. Once cells and treatments were added, the top cover was placed back on the plate and allowed to sit under the hood for 30 minutes. Scans were taken by the xCELLigence machine every 15 minutes for 48 hours. All treatments were performed in triplicate and the number of cells that had passed between chambers (migrated cells) was compared to 1% CS-FBS media only wells and given as a percentage.

mRNA Isolation and Expression

PF or drug-treated EOOF5 cells were suspended in TRI reagent (Cat No: T9424, Sigma-Aldrich, St. Louis, MO) and total RNA was isolated using the manufacturer's instructions. PF or drug-treated eutopic endometrial tissues were homogenized in TRI reagent using zirconium oxide beads in a Bullet Blender® homogenizer (SKU: BBX24, Next Advance, Troy, NY). Supernatant was removed and isolated following manufacturer's instructions. Quantity and quality of RNA were measured in the NanoDrop 2000 spectrophotometer. cDNA synthesis from 1 µg of each sample was performed using iScript cDNA synthesis kit (Cat No: 1708890, Biorad, Hercules, CA). mRNA expression was analyzed in the cDNA samples using SYBR Green (Cat No: 1725270, Biorad, Hercules, CA) and the primers used are listed in Supplementary Table 1.

Protein Expression Using Automated Western Blotting

Protein lysates of the PF and drug treated EOOF5 cells were suspended in RIPA buffer containing protease inhibitors (Cat No: P2714, Sigma-Aldrich, St. Louis, MO). Endometrial tissues were homogenized using zirconium oxide beads in a Bullet Blender® homogenizer (SKU: BBX24, Next Advance, Troy, NY). Protein concentrations were determined using a modified Lowry protocol [211]. 7 µg of protein from either cells or tissues was run on the automated western blotting system WES (Cat No: 004-600, Protein Simple, San Jose, CA). The primary anti-rabbit antibodies for EZH2 (1:50, Cat No: 5426S), H3K27me3 (1:25, Cat No: 9733S) (Cell Signaling,

Danvers, MA), anti-rabbit β-actin (1:100, Cat No: 4870S, Cell Signaling, Danvers, MA), and anti-rabbit H3 (1:100, Cat No: 39451, Active Motif, Carlsbad, CA) were used. HRP-conjugated rabbit secondary antibody provided in the WES kit was used. Plates (12-250 kDa and 25 capillary) were run using default settings and results analyzed using the Compass for WES software. Band area given by the software was used and expressed as their relative ratio to β-actin or H3. Results were expressed as a ratio in which CS-FBS media alone was considered 1. It is important to note that proteins examined using the automated Western Blotting system, WES, will have different expected molecular weights compared to traditional Western blotting, due to differences in technology.

Statistical Analysis

Prism software (version 9.0.0, GraphPad, Inc., La Jolla, CA) was used to perform statistics for all protein and mRNA analyses using one-way ANOVA. P-values less than 0.05 were considered significant.

RESULTS

CXCR4 inhibitor lowers expression of CXCR4 axis and EZH2 in endometrial cells

Ruiz et al had shown that CXCR4 is elevated in endometriotic cell lines, as well as varies to some degree in endometriotic lesions [229], but these studies did not examine regulators of this axis in endometriosis. We looked at whether the peritoneal microenvironment and the inflammatory peritoneal fluid (PF) from women with endometriosis could be causing these changes in expression of the CXCR4-CXCL12-CXCR7 axis. EOOF5 cells were treated with either 1% control (n=8) or endo PF (n=6) alone. PF alone changed the mRNA expression levels of the CXCR4-CXCL12-CXCR7 axis very little. Endo PF alone was shown to increase the expression of *EZH2* nearly 3-fold but did not reach statistical significance. We next tested if a CXCR4

inhibitor, AMD3100, would alter the expression of the CXCR4 axis as well as EZH2 when added alone to the EOOF5 cells. When 1 μM AMD3100 alone was added to the cells (n=3), the expression of all genes showed a trend of lowered expression **(Figure 17A).** mRNA expression of *CXCR4*, *CXCL12*, and *CXCR7* also decreased when cells were treated with AMD3100 in the presence of PF. The greatest decrease in expression was observed in *CXCR7* levels in cells that were treated with 1% endo PF+ 1 μM AMD3100 (0.11-fold, p=0.0196 vs endo PF alone) **(Figure 17B).** Expression of *EZH2* showed a trend in lower expression in the cells treated 1 μM AMD3100 in the presence of PF but none reached significance (**Figure 17C)**.

A

Treatment	CXCR4	CXCL12	CXCR7	EZH2
Control PF	1.23	0.51	1.63	1.29
Endo PF	1.06	0.42	1.23	2.77
1 μM AMD3100	0.42	0.20	0.68	0.77

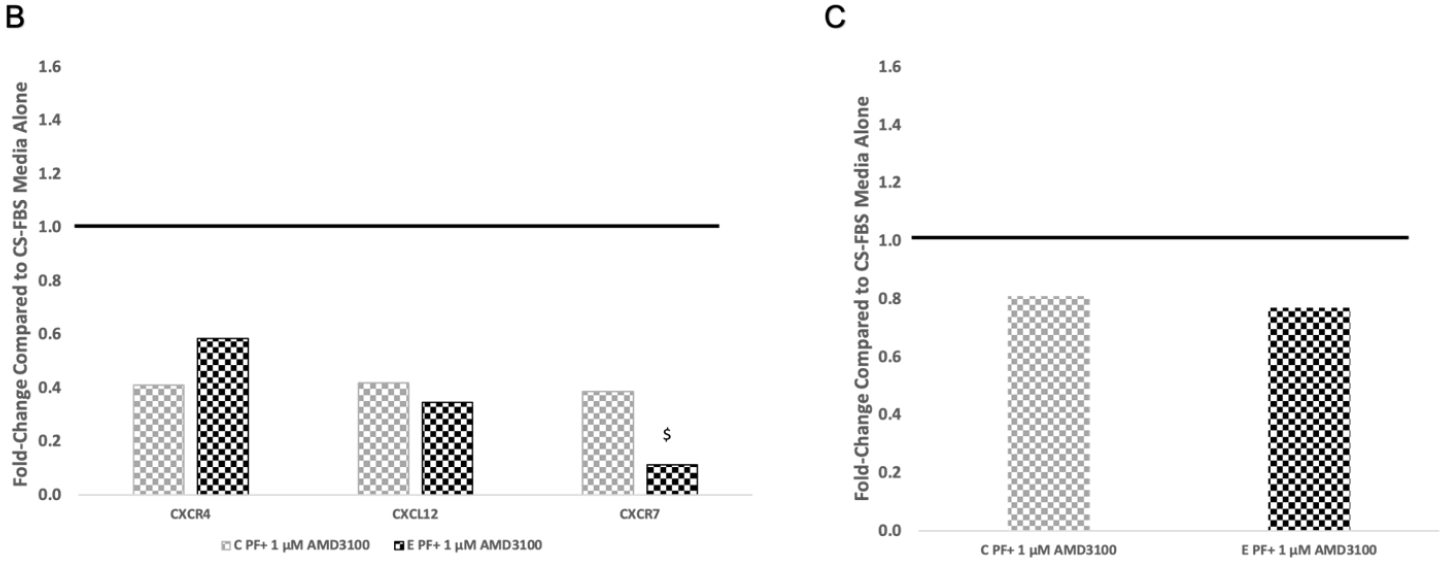


Figure 17: mRNA expression of CXCR4 axis and EZH2 in endometrial cells treated with PF and/or AMD3100 A) Relative mRNA expression of genes examined in PF or AMD3100 alone treatments. EZH2 showed a 2.77-fold increase in expression but was not significant. **B)**

Relative mRNA expression of CXCR4 axis in PF+AMD3100 treated cells. A decrease in all 3 genes was noted and significantly for *CXCR7* (p=0.0196) **C)** Relative mRNA expression of EZH2 showed a decrease in expression. $=significant p-value compared to endo PF alone.

In contrast to the mRNA expression, protein analysis of EOOF5 cells treated with PF or AMD3100 alone showed that expression of EZH2 was increased 1.55-fold (p=0.0221) when endo PF was added to the EOOF5 cells alone. A significant upregulation by 2.03-fold for H3K27me3 was also noted (p=0.0011). When 1 μM AMD3100 alone was added to the EOOF5 cells, expression of both EZH2 and H3K27me3 showed a significant downregulation when compared to both the control and endo PF alone treated cells. EZH2 levels decreased to 0.22-fold (p<0.0001 vs CS-FBS media and 0.0009 vs endo PF) and H3K27me3 to 0.23-fold (p=0.0207 vs CS-FBS media and <0.0001 vs endo PF) **(Figure 18A)**. PF+ 1 μM AMD3100 showed a significant downregulation of EZH2 when added in the presence of both control (0.33-fold) and endo (0.17-fold) PF (p=0.0001 for control PF vs CS-FBS media and <0.0001 vs CS-FBS media or endo PF for all other treatments). H3K27me3 showed a significant downregulation in expression for control PF+ 1 μM AMD3100 (0.11-fold, p=0.0297 vs CS-FBS media alone and <0.0001 vs endo PF alone). Endo PF+ 1 μM AMD3100 also showed a significant downregulation of H3K27me3 (0.24-fold, p<0.0001 vs endo PF alone) **(Figure 18B)**.

A

Treatment	EZH2	H3K27me3
Control PF	$1.30_{+/-0.03}$	$1.84_{+/-0.06}$
Endo PF	$1.55^{*}_{+/-0.007}$	$2.03^{**}_{+/-0.03}$
1 µM AMD3100	$0.53^{**\,\$\$}_{+/-0.015}$	$0.23^{*\,\$\$\$\$}_{+/-0.001}$

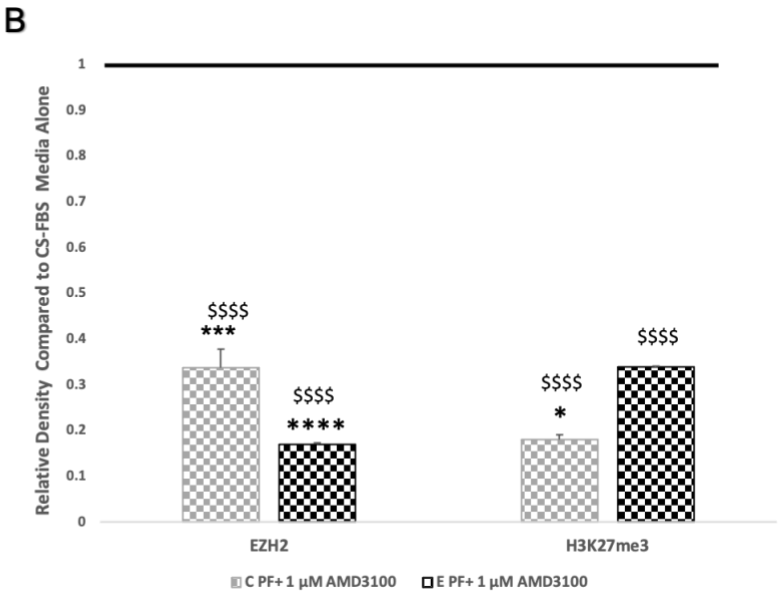


Figure 18: Protein expression of EZH2 and H3K27me3 in endometrial cells treated with PF and/or AMD3100 A) Densitometric analysis of proteins in PF or AMD3100 alone treated cells. EZH2 showed a significant increase of 1.55-fold (p=0.0221) in expression in endo PF treated cells when compared to 1% CS-FBS media alone cells EZH2 decreased significantly when compared to endo PF alone cells when cells were treated with 1 µM AMD3100 alone (0.53-fold, p=0.0221). H3K27me3 also showed a significant increase of 2.03-fold (p=0.0011) in endo PF treated cells as well as when 1 µM AMD3100 alone was added to the cells (0.23-fold, p=0.0207 vs CS-FBS media and p<0.0001 vs endo PF alone). B) EZH2 showed a significant decrease in cells treated with both control or endo PF+ 1 µM AMD3100 when compared to both 1% CS-FBS media alone and endo PF alone cells. Control PF was shown to be 0.33-fold (p=0.0001 vs CS-FBS media and p<0.0001 vs endo PF alone) and endo PF was shown to be 0.17-fold (p<0.0001 vs CS-FBS media and endo PF alone). The expression of H3K27me was shown to be statistically decreased when compared to endo PF alone for both control (0.11-fold) and endo PF (0.24-fold) + 1 µM AMD3100 (p<0.0001) and when compared to CS-FBS media alone for

control PF+ 1 μM AMD3100 (p=0.0297). *=significant p-value compared to CS-FBS media alone. $=significant p-value compared to endo PF alone.

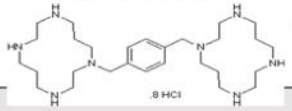
CXCR4 inhibitor increased cell proliferation and decreased migration

Since AMD3100 decreased the mRNA expression of the CXCR4 axis, as well as the mRNA and protein expression of EZH2, its effects on the proliferation of the endometrial cells were determined. The rates of proliferation of the EOOF5 cells treated with PF and/or AMD3100 were detected using the xCELLigence technology. At 48 hours, cells treated with control PF had a 141.5% increase and endo PF had a 142.4% increase in proliferation when compared to media alone cells ($p<0.0001$ for both). At 96 hours, cell proliferation in cells treated with control PF was 126.5% and 128.1% for endo PF treated cells ($p<0.0001$ for both) compared to untreated cells. Surprisingly, the addition of 1 μM AMD3100, actually increased cell proliferation in the presence of peritoneal fluid. At 48 hours, cell proliferation was 190.3% for control PF+ 1 μM AMD3100 ($p<0.0001$) and 180.7% for endo PF+ 1 μM AMD3100 ($p<0.001$) when compared to 1% CS-FBS media alone. At 96 hours, proliferation began to slightly decrease in both control and endo PF+ 1 μM AMD3100, but it was still over 150% for both ($p<0.0001$). Very little change in proliferation was seen in the cells when 1 μM AMD3100 was added alone. When cells were treated with 2.5 μM AMD3100 and control PF a 155.6% increase ($p<0.0001$) in proliferation and with endo PF+ 2.5 μM AMD3100, proliferation increased to 188.6% ($p<0.0001$). Similar to 1 μM AMD3100, proliferation of cells treated with control or endo PF+ 2.5 μM AMD3100 began to decrease at 96 hours but was still significantly higher than CS-FBS media alone with 136.2% ($p<0.0001$) for control PF and 154.97% ($p<0.0001$) for endo PF. In contrast to cells treated with 1 μM AMD3100 alone, cells treated with 2.5 μM AMD3100 alone actually decreased proliferation to 71.2%,

compared to media alone. 2.5 μM AMD3100 alone decreased even further from 48 to 96 hours to 59.5% proliferation (p=0.0107) **(Table 3A)**.

The rate of migration was also examined using xCELLigence technology. We anticipated that the migration rate would be higher when using PF alone based on the results on cell proliferation. For control or endo PF alone, the rate of migration increased to 147% and 180% by hour 6, respectively. These rates began to slowly decrease by hour 12 but were still higher than the CS-FBS media alone showing that PF alone increased migration. Contrary to its effects on proliferation, AMD3100 in the presence of PFs actually decreased migration. While an initial increase was seen for treatments with AMD3100, by hour 6, all treatments were below or around the same rate of the CS-FBS media alone. Both endo PF+ 1 μM AMD3100 and endo PF+ 2.5 μM AMD3100 decreased to 71.66% and 77.59% at hour 9, respectively, and were shown to be statistically significant when compared to endo PF alone (p=0.0080, 0.0202). By hour 12, both PFs lowered migration to 65.56% and 67.63% (p=0.0232, 0.0315 compared to endo PF alone). The biggest decrease in the rate of migration by hour 12 was noted in control PF+ 1 μM AMD3100 at 52% (p=0.0016 compared to endo PF alone) **(Table 3B)**.

A

Treatment	Concentration	24 hours	48 hours	72 hours	96 hours	Outcome
		% Proliferation compared to CS-FBS media alone				
Control PF alone	1%	125.73	141.51****	138.50****	126.47****	Significant increase
Endo PF alone	1%	122.64	142.41****	139.66****	128.11****	Significant Increase
AMD3100 alone 	1 µM	104.88	105.0	110.61	104.81	Proliferation similar to media alone
	2.5 µM	88.26	71.19	63.7	59.54*	Decrease
Control PF+ AMD3100	1% and 1 µM	168.61****	190.3****	185.36****	163.35****	Significant increase
	1% and 2.5 µM	149.21**	155.60****	150.49****	158.77****	Significant increase but not as high as 1 µM
Endo PF+ AMD3100	1% and 1 µM	153.21****	180.75****	181.75****	136.2****	Significant increase
	1% and 2.5 µM	167.11****	188.56****	184.1****	154.97****	More of an increase than 1 µM

B

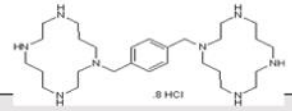
Treatment	Concentration	3 hours	6 hours	9 hours	12 hours	Outcome
		% Migration compared to CS-FBS media alone				
Control PF alone	1%	139.48	147.09	133.77	125.18	Increase
Endo PF alone	1%	161.6	180.03	159.71	145.88	More increase than control PF
AMD3100 alone 	1 µM	106.26	92.24	79.11	74.38	Decrease
	2.5 µM	139.67	116.8	98.72	91.29	Less of decrease than 1 µM
Control PF+ AMD3100	1 µM	89.71	69.69$$$	59.14$$$	51.99$$	Significant decrease
	2.5 µM	120.02	90.55$	64.06$$	54.22$$	Significant decease but not as much as 1 µM
Endo PF+ AMD3100	1 µM	107.51	91.27$	71.66$$	65.56$	Significant decrease but not as much as control PF
	2.5 µM	133.74	104.6	77.59$	67.63$	Significant decrease but not as much as 1 µM

Table 3: Proliferation and migration of endometrial cells treated with PF and/or AMD3100

A) xCELLigence was used to determine the proliferation of the EOOF5 cells in the presence of PF and/or AMD3100. Results at each time point are shown as % proliferation when compared to

CS-FBS media alone cells. The addition of PF to the cells alone significantly increased the proliferation of the cells for the 48-, 72-, and 96-hour time points ($p<0.001$). When AMD3100 was added alone to the cells, proliferation was shown to significantly decrease the proliferation of the cells at 96 hours at the 2.5 μM concentration (59.54%, $p=0.0107$). When AMD3100 was added in the presence of PF, significant increase in proliferation was noted. This increase in proliferation was seen even more with the 2.5 μM AMD3100 concentration than at 1 μM, with endo PF+ 2.5 μM AMD3100 increasing to close to 200% at 48 hours ($p<0.0001$). **B)** Migration of the EOOF5 cells was measured using xCELLigence. Results are given as % migration when compared to CS-FBS media alone cells. While no statistical significance was seen, PF alone increased the migration of the cells, more so in the endo PF treated cells. When AMD3100 was added to the cells, either alone or in combination with PF, significant decreases in migration were noted when compared to endo PF alone. A greater decrease was seen in control PF+ AMD3100 than endo PF+ AMD3100. *=significant p-value compared to CS-FBS media alone. $=significant p-value compared to endo PF alone.

EZH2 inhibition upregulated the PF mediated expression changes of the CXCR4-CXCL12-CXCR7 axis

We recently showed that endo PF can induce EZH2/H3K27me3 in endometriosis [91]. In order to determine if the increased proliferating effects seen with endo PF treatment were due to its activation of EZH2, the above experiments were repeated in the presence of an EZH2 inhibitor, GSK126. EOOF5 cells were treated with 1 or 2.5 μM GSK126 either alone (n=3) or in combination with 1% PF (control (n=6) or endo (n=7)). mRNA analysis revealed that when GSK126 was added to the cells alone, 2.5 μM GSK126 increased the expression of *CXCR4* and *CXCR7*, while slightly

decreasing the expression of EZH2 (**Figure 19A)**. When GSK126 was added in the presence of endo PF, the expression of both *CXCR4* and *CXCR7* increased 4- and 5.15-fold, respectively, but these results were not statistically significant **(Figure 19B)**. When the cells were treated with PF and GSK126, *EZH2* expression was decreased for all treatments when compared to 1% CS-FBS media alone but none reached significance **(Figure 19C).**

A

Treatment	CXCR4	CXCL12	CXCR7	EZH2
1 μM GSK126	0.33	0.45	0.59	1.11
2.5 μM GSK126	2.13	0.82	1.16	0.92

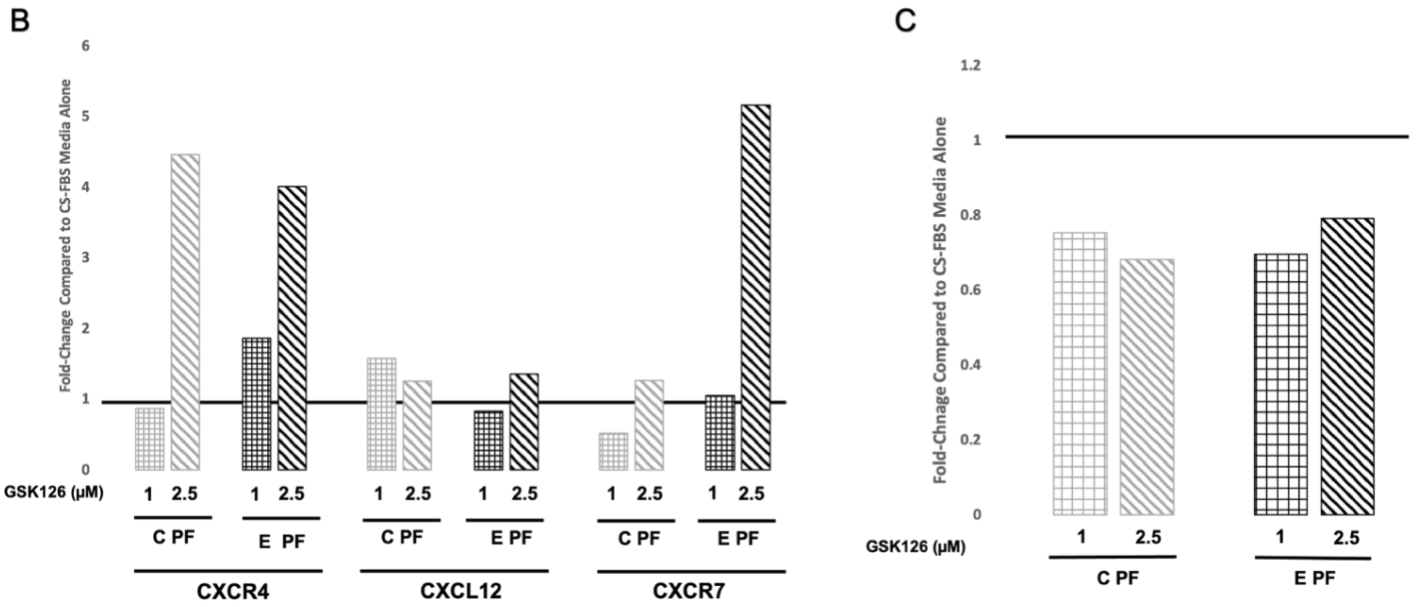


Figure 19: mRNA expression of CXCR4 axis and EZH2 in endometrial cells treated with GSK126 A) Relative mRNA expression in EOOF5 cells treated with GSK126 alone. No significant changes were noted in the expression of the genes examined although an increase of CXCR4 was seen in the 2.5 μM GSK126 alone treatment. **B)** E00F5 cells treated with both PF and GSK126. Both CXCR4 and CXCR7 showed close to or over a 4-fold increase in expression in cells treated with endo PF+ 2.5 μM GSK126 ($p>0.05$). **C)** E00F5 cells treated with PF and GSK126 had lower EZH2 expression but this did not reach significance.

When GSK126 was added to the EOOF5 cells alone, significant downregulation of EZH2 protein expression was seen at both concentrations when compared to endo PF alone treated cells (p=0.0009, 0.0053, respectively). Protein levels of H3K27me3 also showed a lower trend in expression **(Figure 20A)**. When cells were treated with either control or endo PF in the presence of 1 or 2.5 μM GSK126, there was a decrease in the protein levels of EZH2 in all cells treated when compared to CS-FBS media alone cells (**Figure 20B)**. The greatest decrease in EZH2 expression was seen in cells treated with endo PF plus 2.5 μM GSK126 where the fold-change was 0.33-fold (p=0.0334). Control PF+ 2.5 μM GSK126 also significantly decreased the expression of EZH2 when compared to CS-FBS media alone cells (0.44-fold, p=0.0307). All treatments with GSK126 in combination with PF were shown to have a statistically significant downregulation for EZH2 expression when compared to endo PF alone (p<0.0001). When GSK126 was added to the cells in the presence of PF, expression of H3K27me3 was shown to be significantly downregulated when compared to EOOF5 cells treated with 1 μM GSK126 alone. The biggest decrease was seen in cells treated with control PF+ 1 μM GSK126 (0.37-fold, p=0.0297).

A

Treatment	EZH2	H3K27me3
1 µM GSK126	0.87 $$$ +/- 0.05	0.69 +/- 0.23
2.5 µM GSK126	0.91 $$ +/-0.016	0.45 +/- 0.002

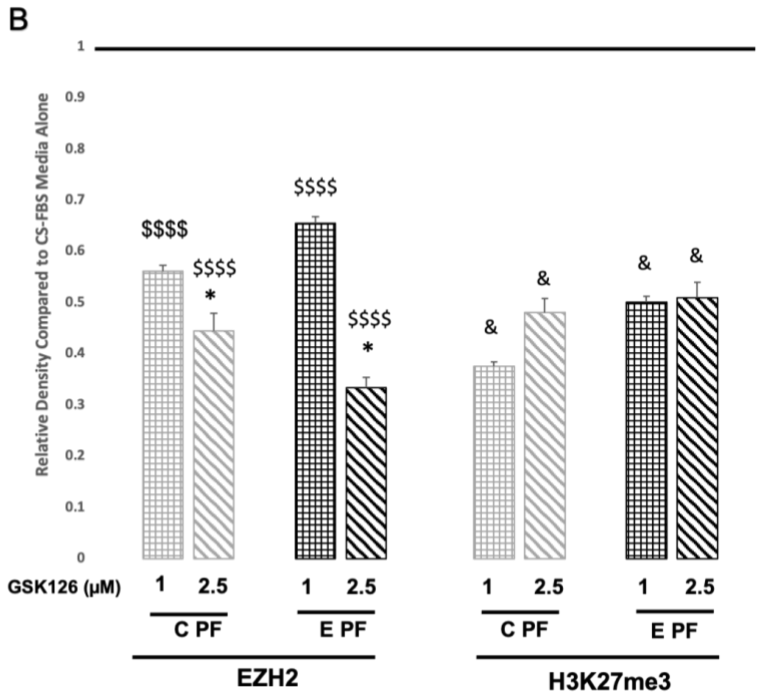


Figure 20: Protein expression of EZH2 and H3K27me3 in endometrial cells treated with GSK126 A) Densitometric analysis of EZH2 and H3K27me3 in cells treated with GSK126 alone. EZH2 showed a significant decrease at both concentrations of GSK126 when compared to endo PF alone (p=0.0009, 0.0053, respectively). **B)** Densitometric analysis of EZH2 and H3K27me3 in EOOF5 cells treated with PF and GSK126. EZH2 showed a significant decrease in all treatments when compared to endo PF alone (p<0.0001). When compared to CS-FBS media alone both control PF and endo PF+ 2.5 µM GSK126 showed a significant decrease in expression (0.44-fold, p=0.0307 and 0.33-fold, p=0.0334, respectively). When compared to 1 µM GSK126 alone, all treatments of PF+ GSK126 showed significant downregulation of expression for H3K27me3. Both concentrations of GSK126 added in the presence of endo PF showed a decrease of approximately half that of CS-FBS media alone cells (0.48-fold, p=0.0271 and 0.51-fold, p=0.0430). *=significant p-value compared to CS-FBS media alone. $=significant

p-value compared to endo PF alone. $=significant p-value when compared to 1 μM GSK126 alone.

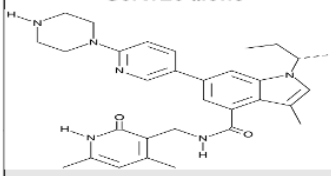
EZH2 inhibitor decreased proliferation but increased migration of endometrial cells

When 1 μM GSK126 alone was added to the cells, proliferation decreased to 55.7% compared to media alone (p=0.0013) at 48 hours. In the presence of PF, proliferation only slightly decreased when compared to 1% CS-FBS media. Proliferation was 99.34% when 1 μM GSK126 was added to control PF and 92.95% when added to endo PF. When 2.5 μM of GSK126 alone was added to the cells, proliferation decreased to 38.9% compared to 1% CS-FBS media (p<0.001). In the presence of PF, proliferation was decreased to 75.7% in the presence of control PF and to 88.01% in the presence of endo PF, but neither were statistically significant. At 96 hours, when 1 μM of GSK126 alone was added to the cells, proliferation decreased even further to 46.44% (p<0.0001). In the presence of PF, proliferation was 61.2% when 1 μM GSK126 was added to control PF (p=0.0067) and 69.9% when added with endo PF (p=0.0002). When 2.5 μM GSK126 alone was added to the cells, proliferation decreased to 36.9% proliferation when compared to 1% CS-FBS media only cells (p<0.0001). In the presence of control and endo PF, 2.5 μM GSK126 decreased proliferation to 61.2% (p<0.0001) and 71.6% (p=0.0025), respectively **(Table 4A)**.

Based on the results of cell proliferation decreasing when GSK126 was added, we anticipated that the migration of the cells would also similarly decrease. However, migration of the cells for all treatments with GSK126 began to significantly increase when compared to CS-FBS media alone before slowly starting to drop or level off at 16 hours. At hour 3, endo PF+ 2.5 μM GSK126 was 242.92% higher compared to CS-FBS media alone (p<0.0001) and was shown to be at a statistically significantly higher rate of migration until hour 10. C PF+ 1 μM GSK126

and E PF+ 1 μM GSK126 were significantly higher when compared to CS- FBS media alone with an increase of 205.98% and 200.63% at 6 hours, respectively (p=0.0075, 0.0161). Even at 12 hours, the rate was 180% higher than that of CS-FBS media alone **(Table 4B)**.

A

Treatment	Concentration	24 hours	48 hours	72 hours	96 hours	Outcome
		% Proliferation compared to CS-FBS media alone				
GSK126 alone 	1 μM	61.6	55.66**	51.52***	46.45****	Significant decrease
	2.5 μM	47.11**	38.87***	36.75***	38.89****	More decrease than 1 μM
Control PF+ GSK126	1% and 1 μM	115.66	99.34	86.08	76.3**	Significant decrease but less than GSK126 alone
	1% and 2.5 μM	91.72	75.73	66.79	61.22****	More decrease than C PF+ 1 μM
Endo PF+ GSK126	1% and 1 μM	111.57	92.95	78.17	67.98****	Significant decrease but less than GSK126 alone
	1% and 2.5 μM	103.88	88.02	78.59	71.62**	Less decrease than C PF+ 2.5 μM

B

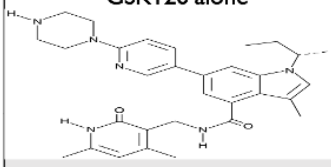
Treatment	Concentration	3 hours	6 hours	9 hours	12 hours	Outcome
		% Migration compared to CS-FBS media alone				
GSK126 alone 	1 μM	130.55	124.63	122.12	120.25	Significant Increase
	2.5 μM	153.10	142.71	137.65	139.9	Greater increase than 1 μM
Control PF+ GSK126	1% and 1 μM	210.44**	205.98**	183.54**	174.93*	Significant increase and more than GSK126 alone
	1% and 2.5 μM	192.17**	177.84	160.3	155.22*	Significant increase
Endo PF+ GSK126	1% and 1 μM	202.9*	200.63*	177.38*	167.98	Significant increase
	1% and 2.5 μM	242.93****	221.76**	189.98**	180.17*	Significant increase and more than C PF+ GSK126

Table 4: Proliferation and migration of endometrial cells treated with GSK126 A) xCELLigence was used to determine the proliferation of the EOOF5 cells when treated with GSK126. Results at each time point are shown as % proliferation when compared to CS-FBS media alone cells. GSK126 added to the EOOF5 cells alone showed a concentration-dependent decrease in % proliferation. When GSK126 was added in the presence of PF, significant decreases were seen by 96 hours but were not as great as when GSK126 was added to the cells alone. **B)** Migration of the EOOF5 cells was determined. While an increase in migration was seen in cells treated with GSK126 alone, none reached significance. At hour 3, cells treated with control PF and 1 μM or 2.5 μM GSK126 had a 210.44% and 192.17% increase, respectively (p=0.0011, 0.0037). Cells treated with endo PF and 1 μM or 2.5 μM GSK126 had a 202.9% and 242.93% increase, respectively (p=0.0124, <0.0001). At hour 12, all treatments except for control PF+ 2.5 μM GSK126 showed significant migration with endo PF+ 2.5 μM GSK126 of 180.17% (p=0.0174). *=significant p-value compared to CS-FBS media alone

Combined inhibition of both CXCR4 and EZH2 provided better overall results

To examine if a combination treatment with AMD3100 and GSK126 would have an even greater effect on lowering the expression of the CXCR4 axis and EZH2/H3K27me3 than each drug individually did, E00F5 cells were treated with a combination of 1 μM AMD3100 and 1 μM GSK126 (alone n=3) or along with control or endo PF n=4). The expression of *CXCR4* and *CXCR7* were slightly increased by the combination drug treatments when compared to the 1 μM AMD3100 alone treatments when the drugs were added without PF. This may be due to the addition of GSK126 in the treatments. The expression of *EZH2* was decreased further in the combination treatment than with AMD3100 alone **(Figure 21A)**. *CXCR4*, expression increased by

1.8-fold when compared to media control cells in cells treated with control PF+ 1 μM AMD3100+ 1 μM GSK126. In contrast, the *CXCL12* expression decreased even more than with 1 μM AMD3100 treatment alone. Endo PF+ 1 μM AMD3100+ 1 μM GSK126 significantly decreased the expression of *CXCL12* with a change of 0.12-fold when compared to media alone (p=0.0385). **(Figure 21B)**. *EZH2* expression decreased from 0.77-fold for endo PF+ 1 μM AMD3100 to 0.32-fold in the endo PF+ 1 μM AMD3100+ 1 μM GSK126 (p=0.0076 vs endo PF alone) **(Figure 21C)**.

A

Treatment	CXCR4	CXCL12	CXCR7	EZH2
1 μM AMD3100 + 1 μM GSK126	0.93	0.26	0.81	0.72

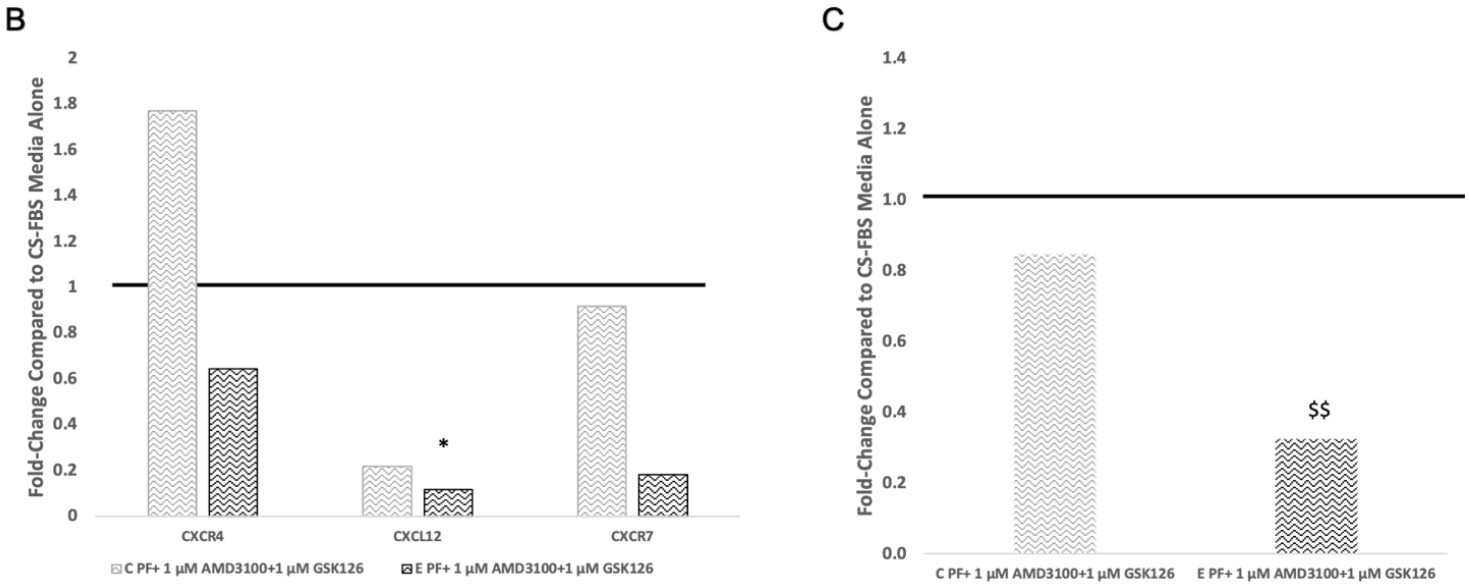


Figure 21: mRNA expression in endometrial cells treated with combination of AMD3100 and GSK126 A) Relative mRNA expression of genes examined in EOOF5 cells treated with 1 μM AMD3100+ 1 μM GSK126 alone. A decrease in expression was noted for all (p>0.05). **B)** Relative mRNA expression of CXCR4 axis in EOOF5 cells treated with PF and 1 μM AMD3100+ 1 μM GSK126. While a slight increase was seen when the combination was added

in the presence of control PF, it was decreased when added in the presence of endo PF for CXCL12 and CXCR4. **C)** Relative mRNA expression of EZH2 decreased in the presence of endo PF. *=significant p-value compared to CS-FBS media alone. $=significant p-value compared to endo PF alone.

Protein levels for EZH2 showed a significant downregulation when 1 μM AMD3100+ 1 μM GSK126 alone was added to the cells when compared to both CS-FBS media or endo PF alone treated cells (0.31-fold, p<0.0001 for both). The expression of EZH2 was shown to decrease even further when the combination was added in the presence of control and endo PF of 0.11- and 0.10-fold, respectfully (p<0.0001 for both when compared to both CS-FBS media alone and endo PF alone cells). The expression of H3K27me3 was also significantly downregulated when cells were treated with control or endo PF in the presence of drug combination. Control PF+ 1 μM AMD3100+ 1 μM GSK126 showed an expression of only 0.11-fold (p=0.172 vs CS-FBS media and <0.0001 vs endo PF). Endo PF+ 1 μM AMD3100+ 1 μM GSK126 was 0.24-fold (p=0.0235 vs CS-FBS media and <0.0001 vs endo PF) **(Figure 22A-B)**.

A

Treatment	EZH2	H3K27me3
1 μM AMD3100+ 1 μM GSK126	$0.31^{****\$\$\$\$}_{+/-\ 0.003}$	$0.54_{+/-0.26}$

B

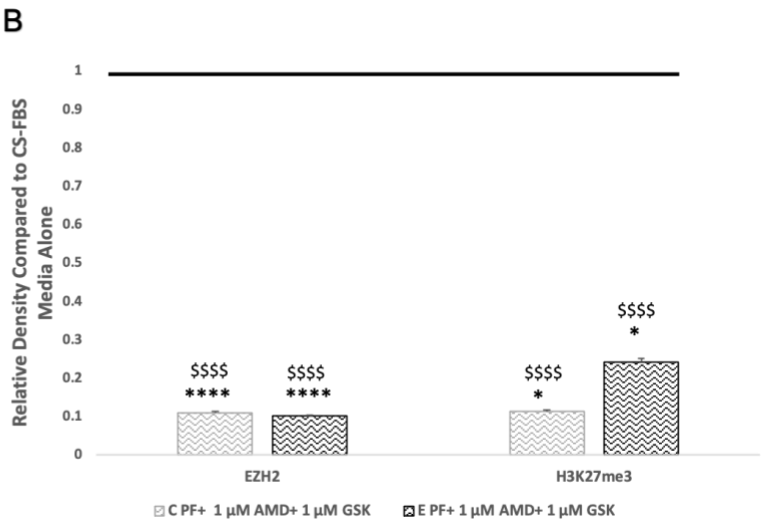


Figure 22: Protein expression of EZH2 and H3k27me3 in endometrial cells treated with combination of AMD3100 and GSK126 A) Densitometric analysis of EZH2 and H3K27me3 in EOOF5 cells treated with 1 μM AMD3100+ 1 μM GSK126 alone. A significant decrease in expression of EZH2 (0.31-fold) was seen when compared to both the CS-FBS media alone cells and the endo PF alone cells ($p<0.0001$ for both). **B)** Densitometric analysis showed a significant decrease in expression of EZH2 when the combination treatment was added in the presence of control or endo PF. For control PF, the relative density of 0.11-fold was seen ($p<0.0001$ compared to both CS-FBS media and endo PF alone). For endo PF a decrease of 0.10-fold was noted ($p<0.0001$ compared to both CS-FBS media and endo PF alone). Expression of H3K27me3 was also significantly downregulated in both the control (0.11-fold) and endo (0.24-fold) PF treatments ($p<0.0001$ when compared to endo PF alone for both and $p=0.0172$, 0.235, respectively vs CS-FBS media alone). *=significant p-value compared to CS-FBS media alone. $=significant p-value compared to endo PF alone.

Both proliferation and migration of cells decreased with use of combination treatment

Using xCELLigence, we examined the effects that the combination of 1 μM AMD3100+ 1 μM GSK126 had on cell proliferation and migration. We anticipated that based on the results of gene expression, both would be decreased. Cell proliferation was shown to initially increase at 48 hours to 143.1% when it was added to control PF and 133.6% when added to endo PF, but neither was statistically significant. However, at 96 hours, proliferation significantly decreased in both control and endo PF+ 1 μM AMD3100+ 1 μM GSK126 to 69.6% ($p=0.0420$) and 61.6% ($p=0.0010$), respectively. When the combination drug treatment was added alone to the cells,

proliferation decreased to 47.8% at 48 hours (0.0046) and 39.8% at 96 hours (p<0.0001) **(Table 5A)**.

The rate of migration was also decreased for treatments with a combination of 1 µM AMD3100 + 1 µM GSK126, but not at as low of a time point as AMD3100. However, by hour 12 both control PF and endo PF+ 1 µM AMD3100 + 1 µM GSK126 were shown to be at a lower rate of migration than CS-FBS media alone at 93.22% and 92.2%, respectively. This was not the case with the treatment of 1 µM AMD3100 + 1 µM GSK126 alone however, as the rate of migration was shown to be statistically significantly decreased when compared to endo PF alone beginning at hour 6 at 72.43% (p=0.0088) and continually decreased. By hour 12, the rate of migration was shown to be 57.71% (p=0.0297 compared to endo PF alone) (**Table 5B)**.

A

Treatment	Concentration	24 hours	48 hours	72 hours	96 hours	Outcome
		% Proliferation compared to CS- FBS media alone				
AMD3100+ GSK126 alone	1 µM for both	49.31*	47.78**	49.89**	39.82***	Significant decrease
Control PF+ AMD3100+GSK126	1 µM for both	143.1	126.01	91.14	69.15*	Significant decrease but not as much as combo alone
Endo PF+ AMD3100+ GSK126	1 µM for both	133.6	113.76	81.04	61.14**	More decrease than C PF+ combo

B

Treatment	Concentration	3 hours	6 hours	9 hours	12 hours	
		% Migration compared to CS-FBS media alone				
AMD3100+ GSK126 alone	1 µM for both	94.03	72.43$$	60.19$	57.71$	Significant decrease
Control PF+ AMD3100+GSK126	1 µM for both	137.91	111.99	100.2	93.22	Initial increase before decreasing
Endo PF+ AMD3100+GSK126	1 µM for both	136.64	115.62	99.41	92.20	More decrease than C PF+ combo

Table 5: Proliferation and migration in endometrial cells treated with combination of AMD3100 and GSK126 A) xCELLigence was used to determine the proliferation of the EOOF5 cells when treated with 1 μM AMD3100+ 1 μM GSK126. A significant decrease in proliferation was seen when the combination was added alone to the EOOF5 cells and was shown to be below 40% proliferation at 96 hours ($p<0.0001$). While an initial increase in proliferation was seen when the combination was added to the cells in the presence of control or endo PF, a significant decrease was noted by 96 hours. Control PF+ combination was 69.15% ($p=0.0420$) and endo PF+ combination was 61.14% ($p=0.0010$). **B)** Migration of the EOOF5 cells was measured using xCELLigence. Results are given as % migration when compared to CS-FBS media alone cells. A significant decrease in migration was seen when the combination treatment was added to the EOOF5 cells alone when compared to endo PF alone migration. While an initial increase in migration was seen when added in the presence of control or endo PF, by hour 12 both treatments were below CS-FBS media alone ($p>0.05$).

EZH2 inhibitor slowed the rate of wound healing while CXCR4 inhibitor increased rate

In addition to examining whether PF, GSK126, or AMD3100 decreases or increases cell proliferation, we wanted to examine if these treatments would increase or decrease the wound healing rate of the cells using a wound/scratch assay. By 72 hours, the scratch for the media alone treatment was completely closed and in some cases by 96 hours the scratch was unrecognizable. For control PF alone, the scratch did not close until 96 hours, compared to endo PF alone which was closed 89.25% at 48 hours and completely at 72 hours. When looking at 1 and 2.5 μM GSK126 alone, the rate of wound healing was shown to be higher than the media alone and the scratches were actually closed by 48 hours. For 1 and 2.5 μM AMD3100 alone, as well as 1 μM GSK126+

1 μM AMD3100 alone, the scratches never fully closed, and cells were actually shown to be floating at the 96-hour time point. For those treatments, the highest percentage of closure was for 2.5 μM AMD3100 at 61% at 72 hours.

Adding GSK126 in combination with PF to the cells was shown to slow the rate of wound healing. For control PF+ 2.5 μM GSK126, the scratch never fully closed at 96 hours (97.6% closure). For all other treatments with PF+ GSK126, no scratches were closed at the 72-hour mark showing that GSK126 slowed the rate of the wound healing when compared to both the time of closure in both the media alone treated cells and the endo PF alone cells.

Adding AMD3100 to the cells in the presence of PF was shown to increase the rate of closure. In fact, for all cell treatments with 1 or 2.5 μM AMD3100 added in the presence of PF, the scratch was shown to close between 48 and 72 hours. Also, control PF+ 1 μM AMD3100 was a 94.18% closure at 48 hours, and both control and endo PF+ 2.5 μM AMD3100 had almost an 85% closure. This is compared to the 55.92% and 72.9% closure seen in the same concentration of GSK126. With cells treated with 1 μM AMD3100+ 1 μM GSK126 in the presence of control PF, the closure of the scratch was significantly low at 24 hours with only a 9.04% closure. This eventually increased and the scratch was closed by 96 hours. For endo PF+ 1 μM AMD3100+ 1 μM GSK126 the rate of closure was lower than that of AMD3100 treatments but was still shown to close by 72 hours. The results for wound healing parallel what was seen for cell proliferation with AMD3100 decreasing migration in the presence of PF while GSK126 addition in the presence of PF increasing this rate **(Figure 23A-B)**.

A

Treatment	0-24 hours	24-48 hours	48-72 hours	72-96 hours
Media Alone	40.82%	83.78%	100%	100%
C PF Alone	38.85%	79.65%	97.67%	100%
E PF Alone	44.09%	89.26%	100%	100%
1 μM GSK126 alone	37.83%	100%	100%	100%
2.5 μM GSK126 alone	59.32%	100%	100%	100%
1 μM AMD3100 alone	40.26%	36.67%	44.40%	Floating cells
2.5 μM AMD3100 alone	52.00%	37.16%	61.00%	Floating cells
1 μM AMD3100+ 1 μM GSK126 alone	44.82%	26.54%	58.30%	Floating cells
C PF + 1 μM GSK126	44.43%	71.66%	80.07%	100%
E PF + 1 μM GSK126	47.73%	66.51%	98.36%	100%
C PF + 2.5 μM GSK126	29.91%	55.92%	87.21%	97.62%
E PF + 2.5 μM GSK126	46.41%	72.93%	97.26%	100%
C PF + 1 μM AMD3100	75.46%	94.18%	100%	100%
E PF + 1 μM AMD3100	52.13%	73.23%	100%	100%
C PF + 2.5 μM AMD3100	60.54%	86.96%	100%	100%
E PF + 2.5 μM AMD3100	47.62%	83.96%	100%	100%
C PF+ 1 μM AMD3100+ 1 μM GSK126	9.05%	78.89%	92.49%	100%
E PF+ 1 μM AMD3100+ 1 μM GSK126	55.94%	76.72%	100%	100%

B

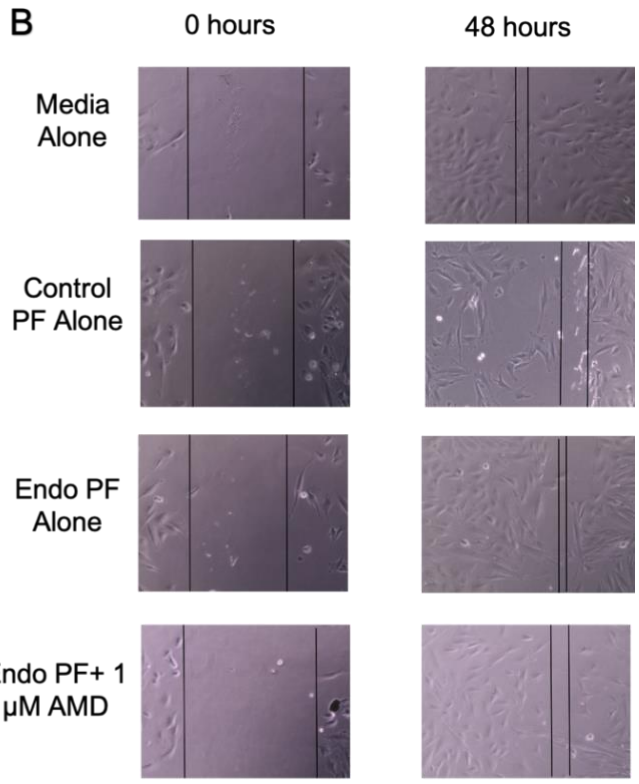


Figure 23: Scratch assay of endometrial cells treated with PF and/or inhibitors A. Table showing the rates of wound healing of the EOOF5 cells in all treatments. 100% indicates completely closed scratch. Overall, when GSK126 was added in the presence of PF, the rate of closure was increased, while AMD3100 lowered that rate slightly and the combination of AMD3100+GSK126 lowered that rate even further. **B.** Representative images of various treatments at 0 hours and 48 hours.

In summary, all the drug treatments taken together (GSK126, AMD3100, and AMD3100+GSK126) showed that while AMD3100 lowered the expression of both the CXCR4 axis and EZH2/H2K27me3, it significantly increased the cell proliferation. While the use of GSK126 decreased proliferation, it increased the expression of CXCR4 as well as increasing the rate of migration, the combination treatment proved to be better overall with decreases in gene

expression as well as in terms of proliferation and migration of the cells. The results presented above also show that the PF from women with endometriosis is having an effect on the way that the drug is interacting with the cells as indicated when looking at the results found when the compounds were added to the cells alone compared to when adding them in the presence of the PF from women with endometriosis.

PF treated eutopic tissue results parallel that seen with PF treated endometrial cells

In order to more fully explain if the changes that were observed for the CXCR4 axis and EZH2/H3K27me3 in isolated primary endometrial cells, would also occur in women with endometriosis, we performed similar studies in eutopic endometrial tissues. These were performed due to the fact that the tissue consists of a heterogeneous cell population, similar to endometriotic lesions, rather than just stromal cells like E00F5. Control and endo PF alone as well as varying concentrations of AMD3100 and GSK126, along with the combination of AMD3100+ GSK126 that was used in the cell treatments, were added to eutopic endometrial tissues (n=3 for all compound alone treatments, n=6 for PF+ compound treatments). When endo PF alone was added to the tissues, we noted an increase in both *EZH2* and *CXCR4* expression by over 2-fold which correlated with what we noted in the cells for *EZH2* but was higher in expression for *CXCR4* when compared to the endo treated cells. All compound alone treatments lowered or kept mRNA expression around the same levels as the tissue alone treatments. *EZH2* was significantly decreased in the 5 μM GSK126 alone treatment when compared to endo PF alone (0.27-fold, p=0.0467 vs endo PF alone) **(Figure 24A)**. qPCR analysis showed that when eutopic tissues were treated with 1 or 2.5 μM AMD3100 in the presence of PF, the CXCR4-CXCL12-CXCR7 axis showed a decrease in mRNA expression. The largest decreases in expression were seen in *CXCR7* with endo

PF+ 1 μM AMD3100 being lowered to 0.39-fold (p=0.0208). The only exception was *CXCL12* in the endo PF+ 2.5 μM AMD3100 treatment which showed a 1.16-fold increase in expression, but this was not significant **(Figure 24B)**. Expression of *EZH2* was actually shown to increase when 2.5 μM AMD3100 was added in the presence of endo PF to 1.31-fold but this was not significant **(Figure 24C)**.

Tissues treated with endo PF and GSK126 showed a significant increase in *CXCR4* for the 3, 5, and 9 μM GSK126 treatments by over 5-fold for all three concentrations (p=0.0122, 0.0301, and 0.0143, respectively). 1 μM GSK126 in the presence of endo PF also showed a 3.5-fold increase in expression of *CXCR4*, but this was not statistically significant. For *CXCL12* and *CXCR7*, a significant up-regulation was seen in the endo PF+ 9 μM GSK126 treatments with a 3.84-fold increase for *CXCL12* (p=0.0261 compared to tissue alone) and 3.67-fold for *CXCR7* which was shown to be statistically significant when compared to both tissue alone and endo PF alone (p=0.0079, 0.0089, respectively). Expression was also increased for the 3 μM addition in the presence of endo PF, but these results were not statistically significant. While upregulation was also seen in tissues treated with control PF+ GSK126, none were shown to be statistically significant **(Figure 24D)**. *EZH2* expression was lowered at all concentrations of GSK126 added in the presence of PF when compared to endo PF alone tissues and was significantly downregulated for 1 and 5 μM GSK126 treatments in the presence of endo PF (p=0.0070 and 0.0044, respectively) **(Figure 24E)**.

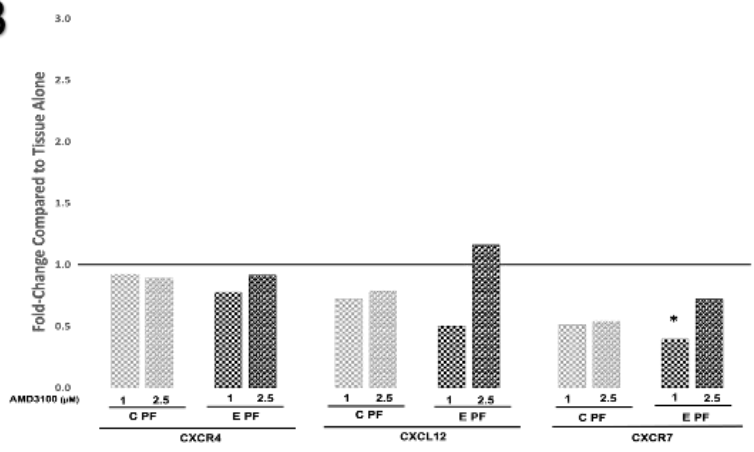
The combination of 1 μM AMD3100+ 1 μM GSK126 showed a downregulation of the CXCR4-CXCL12-CRCR7 axis with a greater decrease in expression seen when added in the presence of endo PF vs control PF, with all treatments showing an expression less than the tissue

alone. However, none showed statistical significance. EZH2 expression showed little change in expression in the PF+ AMD3100+ GSK126 treatments **(Figure 24 F-G)**.

A

Treatment	CXCR4	CXCL12	CXCR7	EZH2
Control PF	1.23	0.69	0.60	1.17
Endo PF	2.00	1.04	0.88	2.11
1 μM AMD3100	0.98	0.46	0.24	0.67
2.5 μM AMD3100	1.84	0.68	1.14	1.12
1 μM GSK126	2.70	1.37	1.11	0.49
3 μM GSK126	1.84	0.68	1.14	1.12
5 μM GSK126	1.88	0.97	0.76	0.27 $
9 μM GSK126	0.77	0.64	0.59	0.52
1 μM AMD3100 + 1 μM GSK126	0.87	1.07	0.76	0.68

B



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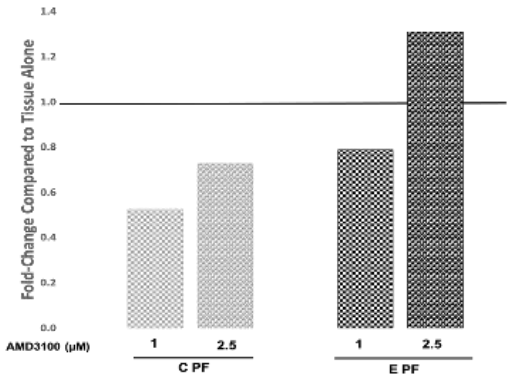

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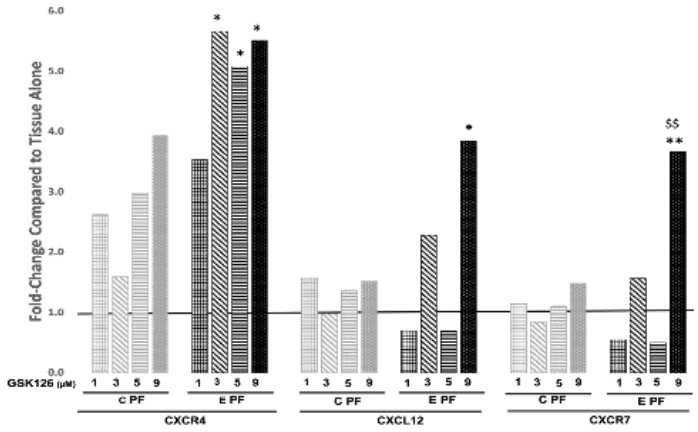

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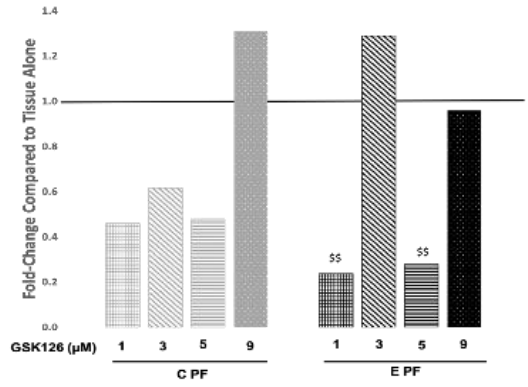

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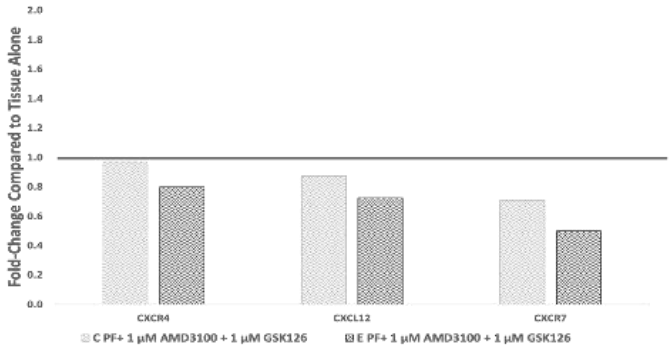

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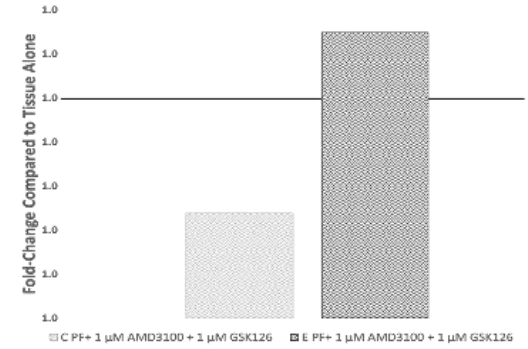


Figure 24: mRNA expression of CXCR4 axis genes and EZH2 in endometrial tissues treated with PF and/or inhibitors A. mRNA expression when PF or compounds were added to eutopic endometrial tissues alone paralleled the results noted with similar treatments with endometrial cells. The only statistical significance noted was for the expression of *EZH2* in the 5 μM GSK126 alone treatment (0.27-fold, p= 0.0467 vs endo PF alone tissues). **B.** mRNA expression of the CXCR4 axis showed a decrease in expression, with *CXCR7* showing a significant decrease when 1 μM AMD3100 was added in the presence of endo PF (0.39-fold, p=0.0208). **C.** mRNA expression of *EZH2* in AMD3100 treated tissues. EZH2 expression increased in the endo PF+2.5 μM AMD3100 treated tissues, while all other treatments decreased the expression. **D.** mRNA expression of the CXCR4 axis showed a significant increase in expression when GSK126 was added in the presence of endo PF. *CXCR4* was shown to be significantly increased at the 3, 5, and 9 μM GSK126 additions by over 5-fold (p=0.0122, 0.0301, and 0.0143, respectively). *CXCL12* and *CXCR7* were also shown to be significantly increased in the 9 μM additions to the tissues in the presence of PF (3.84-fold, p=0.0261 and 3.67-fold, p=0.0079 vs tissue alone and p=0.0089 vs endo PF alone tissue). **E.** mRNA expression of *EZH2* showed a significant decrease in expression of *EZH2* when 1 and 5 μM GSK126 was added to the tissues in the presence of endo PF (p=0.0070, p=0.0044). **F.** mRNA expression of the CXCR4 axis when 1 μM AMD3100+ 1 μM GSK126 was added in the presence of PF to the tissues. While no significant p-values were noted, the expression of all 3 genes was below the expression of tissues alone. **G.** mRNA expression of *EZH2* when 1 μM AMD3100+ 1 μM GSK126 was added to the tissues in the presence of PF showed no increase or decrease in *EZH2* expression (p>0.05). *=significant p-value compared to CS-FBS media alone. $=significant p-value compared to endo PF alone.

Protein expression of EZH2 was significantly increased in endo PF treated tissues compared to untreated tissue (2.19-fold, p=0.0348) **(Figure 25A)**. An increase in expression of H3K27me3 was also noted in the endo PF treated tissues correlating with what was previously seen in the endo PF treated cells. When 1 μM AMD3100 alone was added to the tissues, H3K27me3 showed a significant downregulation when compared to endo PF alone treated tissues (0.61-fold, p=0.0231). Significant downregulation when compared to endo PF alone tissues was also seen for H3K27me3 for the 3 μM and 5 μM GSK126 alone treated tissues (0.46-fold, p=0.0495 and 0.20-fold, p=0.0109, respectively). EZH2 also showed a significant decrease in expression tissues in the 1 μM and 5 μM GSK126 alone treatments (0.71-fold, p=0.0082 and 0.81-fold, p=0.0178, respectively) when compared to endo PF alone.

Protein expression of tissues treated with PF+ AMD3100 confirmed the results seen for the mRNA. Both concentrations added in the presence of endo PF showed a significant downregulation of EZH2 by 0.18- and 0.20-fold when compared to endo alone tissues (p=0.0016, 0.0004). Expression of H3K27me3 was also significantly downregulated in the tissues treated with endo PF+ 1 μM AMD3100 when compared to the endo PF alone tissues (0.89-fold, p= 0.0037) as well as when 2.5 μM AMD3100 was added in the presence of control or endo PF when compared to endo PF alone tissues (0.79-fold, p=0.0238 and 0.67-fold, p=0.0171, respectively) **(Figure 25B)**.

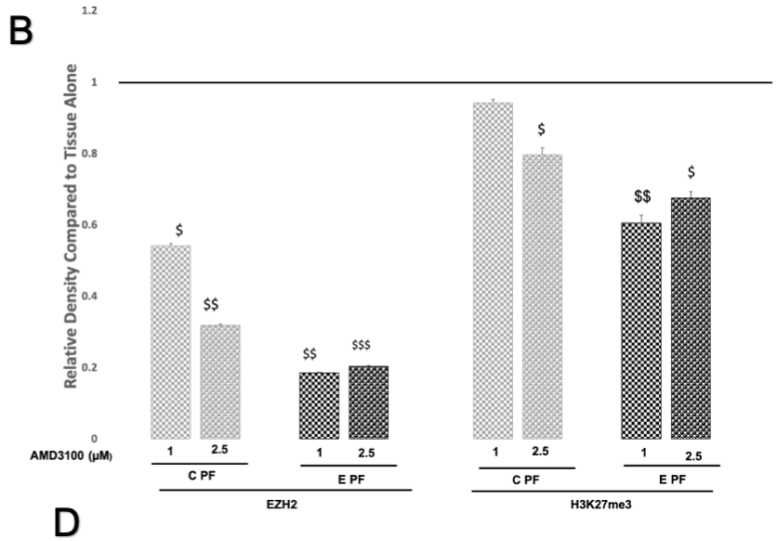
Eutopic tissues treated with all concentrations of GSK126 (1, 3, 9, 9 μM) in the presence of control or endo PF showed a significant downregulation of EZH2 expression when compared to tissue alone. E PF+ 5 μM GSK126 showed a decrease in expression by 0.54-fold (p<0.0001). E PF+ 9 μM GSK126 showed the highest decrease in expression by 0.47-fold (p=0.0002). Tissues treated with control PF plus 1 or 5 μM GSK126 showed a significant downregulation of

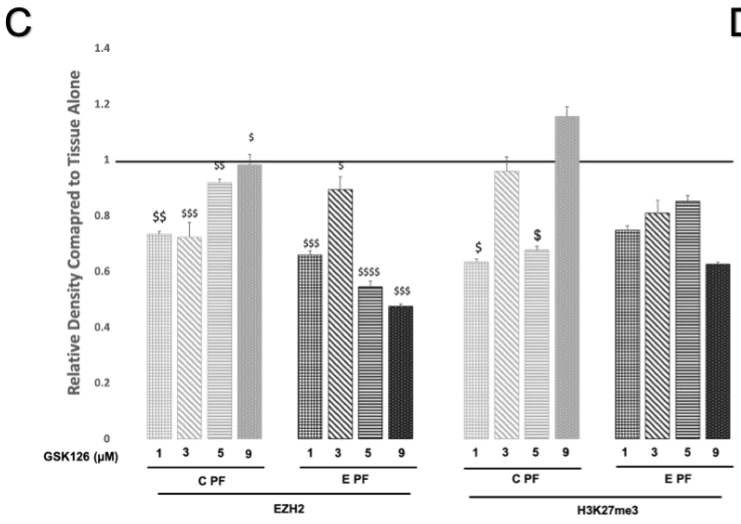
H3K27me3 when compared to endo PF alone (0.63-fold, p=0.0273 and 0.68-fold, p=0.0365). **(Figure 25C)**.

Lastly, protein expression of EZH2 and H3K27me3 was examined in the combination treatment of 1 μM AMD3100+ 1 μM GSK126. EZH2 expression was shown to be significantly decreased in expression when the combination was added in the presence of both control PF (0.20-fold) and endo PF (0.40-fold) (p=0.0004, 0.0031 compared to endo PF alone). The expression of H3K27me3 was also shown to be significantly downregulated in the tissues treated with endo PF+ 1 μM AMD3100+ 1 μM GSK126 when compared to the tissues treated with endo PF alone (0.69-fold, p=0.0029). **(Figure 25D)**.

A

Treatment	EZH2	H3K27me3
Control PF	$1.53_{+/-0.002}$	$0.80_{+/-0.04}$
Endo PF	$2.19^{*}_{+/-0.002}$	$2.14_{+/-0.04}$
1 μM AMD3100	$1.01_{+/-0.003}$	$0.61^{\$}_{+/-0.03}$
2.5 μM AMD3100	$0.75_{+/-0.004}$	$0.76_{+/-0.006}$
1 μM GSK126	$0.71^{\$\$}_{+/-0.02}$	$0.99_{+/-0.02}$
3 μM GSK126	$1.01_{+/-0.004}$	$0.46^{\$}_{+/-0.002}$
5 μM GSK126	$0.81^{\$}_{+/-0.002}$	$0.20^{\$}_{+/-0.005}$
9 μM GSK126	$1.13_{+/-0.002}$	$0.65_{+/-0.01}$
1 μM AMD3100+ 1 μM GSK126	$0.75_{+/-0.006}$	$0.88_{+/-0.08}$





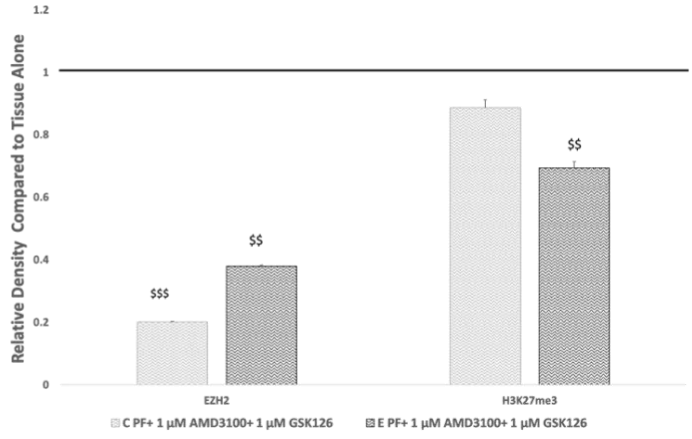


Figure 25: Protein expression in endometrial tissues treated with PF and/or inhibitors A. Densitometric analysis of the protein expression when PF, compounds, or combination were added to the tissues alone showed a significant increase in EZH2 (2.19-fold, p=0.0348) in the endo PF treatments. H3K27me3 was also shown to increase but was not significant. When 1 μM AMD3100 was added, H3K27me3 decreased to 0.61-fold (p=0.0231). EZH2 showed significant downregulation in the 1 and 5 μM GSK 126 additions of 0.71-and 0.81-fold, respectively (p=0.0082, 0.0178, respectively vs endo PF alone tissues). A significant decrease in expression of 0.20-fold (p= 0.0495 vs. endo PF alone tissues) was also seen at 5 μM GSK126 for H3K27me3 as well as at the 3 μM GSK126 addition (0.46-fold, p= 0.0109 vs endo PF alone tissues). **B.** Densitometric analysis of EZH2 and H3K27me3 in tissues treated with AMD3100 in the presence of PF showed a significant decrease when compared to endo PF alone for both proteins in all treatments. The only exception was the expression of H3K27me3 in the control PF+ 1 μM AMD3100 addition, but expression was still below 1. **C.** Densitometric analysis of EZH2 and H3K27me3 in tissues treated with GSK126 in the presence of PF showed a significant decrease in the expression of EZH2 in all treatments when compared to the expression of endo PF alone tissues, with all being shown to be below 1. The expression of H3K27me3 was shown to be significantly decreased in the 1 and 5 μM additions to the tissues in the presence of control PF when compared to endo PF alone (0.79-fold, p=0.0238 and 0.67-fold, p=0.0171, respectively). Additions of GSK126 at all concentrations in the presence of endo PF showed decreased expression of H3K27me3 as well but none were shown to be significant (p>0.05). **D.** Densitometric analysis of EZH2 and H3K27me3 in tissues treated with 1 μM AMD3100+ 1 μM GSK126 in the presence of PF showed significant decreases in the expression of EZH2 when added in the presence of control PF (0.20-fold) and endo PF (0.40-fold) (p=0.0004, 0.0031 vs

endo PF alone). H3K27me3 showed a significant decrease in expression of 0.69-fold when the combination was added in the presence of endo PF (p=0.0029 vs endo PF alone tissues). *=significant p-value compared to CS-FBS media alone. $=significant p-value compared to endo PF alone.

DISCUSSION

Endometriosis is a complex disease with multiple suggested theories of its origin. One commonality among all these theories is that inflammation plays a major role in its etiology. Inflammatory molecules are shown to be highly upregulated in endometriosis, especially in the peritoneal fluid [16,17,19-23]. We recently showed that the PF from women with endometriosis is also able to induce epigenetic changes, specifically in the expression of the PRC2 complex and its catalytic subunit, EZH2 [91]. Though other studies in the literature have also seen upregulation of EZH2 in endometriosis [88,89,93,164] the mechanism(s) by which these epigenetic pathways are modulated in endometriosis is not currently known and was the focus of this study. A knowledge of the underlying mechanisms will assist in validating the use of epigenetic inhibitors for treatment of endometriosis. The present study confirmed our previous results as the primary endometrial stromal cells (EOOF5) showed a significant increase in protein expression of EZH2 of 1.55-fold when treated with endo PF alone (p=0.0221). This increase in EZH2 protein expression was also seen in eutopic tissues treated with endo PF (2.19-fold (p=0.0348)). As EZH2 activates H3K27me3, its expression was also shown to be significantly upregulated 2.03-fold in the EOOF5 cells treated with endo PF (p=0.0011). We also noted that the use of both control and endo PF increased the proliferation and migration of the cells. This similarity between using the two types

of PF may be related to the source of the samples (patients having similar inflammatory molecules in the PF, due to underlying clinical condition).

One key inflammatory pathway that is shown to be upregulated in endometriosis is the CXCR4 axis. The CXCR4 axis can control migration, as well as invasion and proliferation of cells [240]. Ruiz et al. showed that the nuclear levels of CXCR4 are higher in ectopic lesions when compared to proliferative endometrium from controls and that this was even higher in the stromal compartment and in ovarian lesions. In an endometriotic cell line (12Z), a higher expression in CXCR4 also led to an increase in proliferation when compared to control cells, but a higher expression of CXCL12 did not increase the proliferation rate [229]. In the present study, though the mRNA expression of CXCR4/CXCL12/CXCR7 did not significantly increase in endo PF treated cells, the expression of *CXCR4* was increased over 2-fold in the endo PF treated eutopic tissues showing that PF can induce this axis.

Due to initial evidence for its role in endometriotic lesion formation, it was suggested that targeting CXCR4 in women with endometriosis might provide a new non-hormonal treatment [229]. We tested if the CXCR4 inhibitor, AMD3100 would inhibit or reverse the PF mediated effects that were noted in the epigenetic mechanisms of EZH2. We hypothesized that by targeting these PF mediated epigenetic changes in CXCR4, we could provide a more tailored treatment for endometriosis, lowering the pain and lesion size. Using AMD3100, we noted that the mRNA expression of *CXCR4*, *CXCL12*, and *CXCR7* was decreased in expression. AMD3100 was also able to decrease the expression of EZH2 and H3K27me3. We thus anticipated that AMD3100 would also decrease cell proliferation and migration. While migration did not significantly decrease, proliferation of the cells was shown to significantly increase when AMD3100 was added in the presence of endo PF. These results differed from the Pluchino, et al. study where AMD3100

had no significant effect on proliferation of endometrial cells [227]. However, when AMD3100 was added in presence of PF, proliferation of E00F5 was significantly increased. This finding shows that factors in the peritoneal fluid are blocking the effects of AMD3100.

In endometrial cancer, 1-10 μM GSK126 suppressed the cancer cell growth and decreased the number of cancer cell colonies [239]. We recently showed that endo PF can induce the EZH2 pathway in endometriosis [91]. With this knowledge, we wanted to examine if blocking EZH2 would also block CXCR4 axis and hence block PF influence on the CXCR4 mediated effects. We saw that when GSK126 was used in the presence of endo PF, it changed the behavior of the compound. Through the studies presented here, we found an upregulation of the CXCR4 axis in the endometrial cells and tissues treated with PF and GSK126, while GSK126 significantly decreased the mRNA and protein expression of EZH2 in the endometrial cells and tissues in the presence of PF. Protein expression of H3K27me3 was also shown to be significantly decreased in expression when GSK126 was used in the presence of PF in both the cell and tissue model. These results correlated with studies in other diseases that have shown that while GSK126 does decrease the levels of EZH2/H3K27me3, which was also seen here, it also increases the levels of CXCR4 and other inflammatory markers due to increased signaling through NF-kB and genes residing in PRC2-regulated chromatin [237,241].

As seen with AMD3100, performing proliferation and migration studies using GSK126 on the endometrial cells also showed dual results, as also noted when using AMD3100. GSK126 was shown to lower the proliferation of the cells. However, this decrease in proliferation was not as significant as when GSK126 was added to the cells alone. When looking at migration of the cells though, it significantly increased the rate of migration and even more so in the presence of PF than

when added alone. Further suggesting that the PF is having an effect on the cells and may be causing GSK126 to not work to its full potential.

Due to the opposing results that AMD3100 and GSK126 had on the PF mediated effects on the CXCR4 axis and EZH2, as well as on proliferation and migration of endometrial cells, we tested both in a combination approach (1 μM AMD3100+ 1 μM GSK1216), targeting both inflammation and epigenetics. Overall, this approach resulted in more positive results. While mRNA expression was shown to be higher in the combination treatment than the treatments with just AMD3100, they were not significantly increased for what we noted for the GSK126 treatments for the CXCR4 axis. For all 3 genes in the CXCR4 axis, the expression was shown to be below 1 when the combination treatment was added in the presence of endo PF to the eutopic tissues. The protein expression of both EZH2 and H3K27me3 was also shown to be significantly decreased when this combination was added in the presence of endo PF. We also noted that this combination significantly lowered the cell proliferation and while it initially increased the migration, the rate was below the CS-FBS media alone cells by hour 12.

In summary, we noted that when adding endo PF to the cells alone, this increased the expression of EZH2/H3K27me3 but did not increase the expression of CXCR4 as we were expecting. It has been shown in other studies that there are repressive marks for H3K27me3 on the CXCR4 promoter, inhibiting its expression [237]. From these studies, that appears to be true in our studies as well. The increase in expression of H3K27me3 in endo PF treated cells and tissue inhibited the CXCR4 expression in those same treatments. It is unclear how AMD3100 affects EZH2. It may due to its effect on CXCR4. By reducing the expression of CXCR4, decreases in inflammation and inflammatory molecules present in the PF would be noted, which in turn would not be able to upregulate EZH2/H3K27me3 expression as was seen when endo PF alone was

added. Other studies have noted that the use of AMD3100 decreased the components of the PRC1 complex [242]. In a model of PRC recruitment, it has been proposed that methylation of H3K27me3 due to PRC2 is able to recruit PRC1, which in turn facilitates the formation of heterochromatin and repression of genes [71] While its effects haven't been studied on the PRC2 complex in the same manner, it may also be working the same way as noted with PRC1 as both PRC2 and PRC1 have similar function of shutting down gene transcription. While it was suggested that the use of AMD3100 would reduce the pain that these women experience due to the decrease that we noted of both the CXCR4 axis and EZH2, the significant increase in proliferation was noted when AMD1300 was used, especially in the presence of endo PF, suggests that this would not be a good option to reduce the size of the lesions that are present. On the other hand, the use of GSK126 decreased the expression of H3K27me3, which may have released those repressive histone marks on CXCR4, allowing for full expression of CXCR4. This significant increase in the expression of CXCR4 may also be due to the fact that we are now able to see the expression that we expected when endo PF was added alone. This also now allows CXCR4 to activate inflammatory pathways in the peritoneal cavity, thus releasing factors such as VEGF and MMP-9, both of which have been shown to be upregulated and playing a role in endometriosis [42,44,243-245]. Noting the decrease in proliferation with the use of GSK126, this would be a good treatment for targeting the endometriotic lesions, but the increase in the CXCR4 axis would not target the inflammation and pain that these women experience. The overall best results were observed using the dual targeting of CXCR4 (AMD3100) and EZH2 (GSK126). By hitting this mechanism from both angles, we were able to restrict both the inflammatory pathway and epigenetic pathway and not cause debilitating changes to one or the other. This treatment option may be the way to target both the lesion size, as well as the pain that affected women experience **(Figure 26)**.

All of these results together show that there is more interplay between inflammation and epigenetic mechanisms than we originally thought. While GSK126 has begun to be examined in other laboratories as a treatment for endometriosis, our findings should serve as a caution since we have shown that in the presence of PF from women with endometriosis, GSK126 significantly changes the expression of other genes such as CXCR4 which is known to increase migration/invasion in cancer. This is important as CXCR4 has been shown to be highly upregulated in ovarian cancer. With 2.9% of women with endometriosis developing ovarian cancer, this risk of transformation could be increased if GSK126 was used as a treatment due to the upregulation of CXCR4. This is a likely scenario as shown through our migration assay. The results shown here point towards a combination approach of targeting both the epigenetic genes and the chemokine genes as a more successful and more tailored treatment for women afflicted with endometriosis, as well as lowering the probability of developing ovarian cancer.

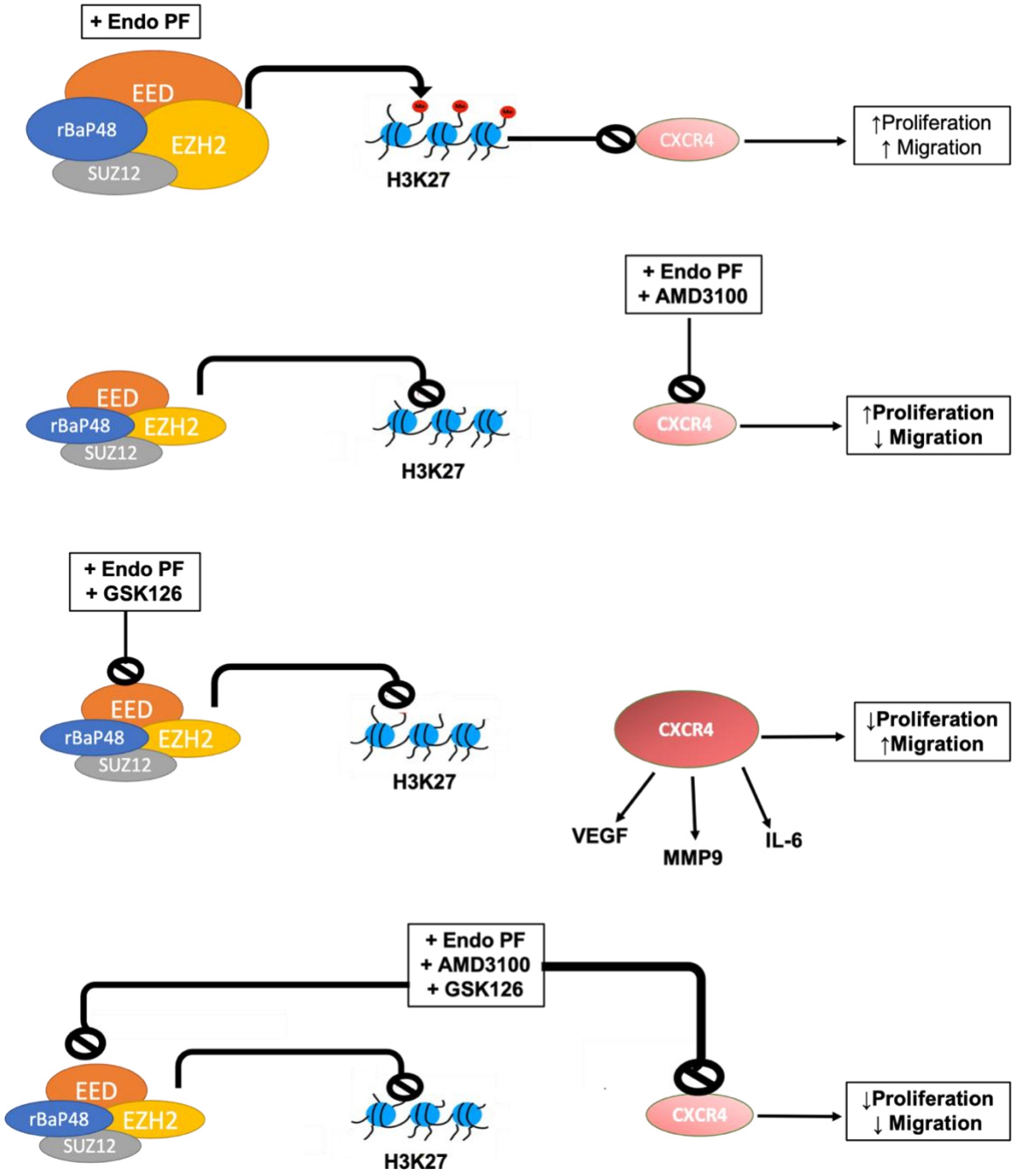


Figure 26: Schematic summarizing results and mechanisms noted throughout the study

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Gene	Sequence
18S	F: GCAATTATTCCCCATGAACG
	R: GGCCTCACTAAACCATCCAA
EZH2	F: AAGGAGTTTGCTGCTGCTCT
	R: ATTAATGGTGGGGGTGCTGG
CXCR4	F: CCAGCGCTGAAGCTGAAGA
	R: GTCCGGCCTTGATCACCTC
CXCL12	F: GGACTTTCCGCTAGACCCAC
	R: GTCCTCATGGTTAAGGCCCC
CXCR7	F: TCTGGGAGACGAATCCTGCT
	R: TCCTTCAAGTTCCCAGACCC

Supplementary Table 2: Primer sequences for RT-qPCR analysis of gene expression in treated cells and tissues. Primers were designed using NCBI GenBank and ordered from Invitrogen.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The goal of this thesis was to explore the interplay between inflammation and epigenetic mechanisms involved in endometriosis. In particular, the focus was on the inflammatory pathways that regulate the PRC2 complex and EZH2. We also wanted to explore the role of peritoneal fluid (PF) from women with endometriosis as one of the key modulators of the epigenetic events that are observed in endometriosis. Previous studies performed in our laboratory showed that the PF from women with endometriosis has increased inflammatory factors, and was able to differentially regulate epigenetic molecules (microRNA) in endometrial cells compared to the PF from women without endometriosis [16,17,21,22]. While this previous work showed the effects that the PF has on the molecules responsible for the pain that women with endometriosis experience, the studies presented in this thesis took those studies further and explored the effect that the PF had on the epigenetic mechanisms and the lesions that are present in these women.

The introductory Chapter 1 provided background literature on endometriosis, its pathology, and current treatments. Literature showed an abundance of Inflammatory and oxidative stress markers in the endometriotic PF. There are suggestions of epigenetics playing a role in the etiology of endometriosis. There is plausible evidence of interplay between inflammatory molecules and epigenetic mechanisms that have been shown to be at work in endometriosis, though this has not been previously explored. It is also still unclear if epigenetic mechanisms are affecting the inflammatory pathways or the inflammatory milieu are modulating the epigenetic mechanisms at work or if this is a vicious cyclic loop. Uncovering this interplay is critical in providing new and more tailored treatments for women with endometriosis.

Chapter 2 is the first study of the thesis that explores the mechanisms driving the PRC2 complex regulation in endometriosis. We hypothesized that there was a complex interplay between the epigenetic factors (miR-155, PRC2 complex, JARID2) and the inflammatory mediator FOXP3. Though initially it was predicted that the PF from women with endometriosis was increasing the expression of JARID2 which was acting as the helper for the PRC2 complex, our studies found that though this was partially true, we also found that a microRNA and known inhibitor of JARID2, miR-155, was also increased in tissues from women with endometriosis, as well as endo PF treated cells. Performing gain-or-loss- of function studies for miR-155 had no effect on the expression of JARID2 suggesting that in endometriosis miR-155 is not suppressing the expression of JARID2 directly but rather indirectly not allowing it to perform its normal function of helping bring the PRC2 complex to its target. Further studies showed that another epigenetic protein, PHF19, was highly upregulated in endo PF treated cells. This is an important finding since PHF19 has been shown to work along with miR-155/FOXP3 and this complex together performs the same function as JARID2. This finding explained how miR-155 regulated the function of JARID2. Overall, this study showed that the PF from women with endometriosis and its inflammatory environment is having an effect on the miR-155-PRC2 complex-JARID2 cross-talk seen at work in endometriosis, as well as providing alternative targets (JARID2, miR-155 or PHF19) for treatment options [91].

Chapter 3 is the second study of the thesis, which looked at whether the effects of the epigenetic mechanisms presented in Chapter 2 were inhibited and if this would be able to produce a tailored treatment for endometriosis. Specifically, we wanted to see the effects on the PRC2 complex if we inhibited an inflammatory pathway. The thought was that the inflammatory molecules are having an effect on these epigenetic mechanisms and if we can inhibit those

inflammatory pathways, we could inhibit or decrease the epigenetic mechanisms. We chose the CXCR4-CXCL12-CXCR7 axis as CXCR4 and EZH2 have been shown to have correlation in expression levels [235-237]. When inhibiting the CXCR4-CXCL12-CXCR7 axis (AMD3100) in the presence of endometrial PF, dual results were seen in both PF treated normal eutopic tissue and primary endometrial cell model. While we did get a decrease in the gene expression of the CXCR4 axis, as well as EZH2, in both the cells and the tissue as well as the migration of the cells, there was a significant increase in the proliferation of the cells. Due to these unexpected results, we looked at inhibiting EZH2 (GSK126), the epigenetic mechanisms, in hopes of uncovering what is actually affecting this response in endometriosis. In both the PF treated tissue and the cell model, GSK126 treatment as expected decreased in the expression of EZH2 and H3K27me3 and cell proliferation. However, contrary to expectations, there was a significant increase in the expression of the CXCR4-CXCL12-CXCR7 axis as well as a significant increase in cell migration. This forced us to look at the pathway from a third angle and provide a combination therapy blocking both the inflammatory pathway (CXCR4 axis) as well as the epigenetic mechanisms (EZH2 pathway). The use of this combination approach (AMD3100 + GSK126) provided us with the best overall results in terms of decreasing gene expression as well as decreasing cell proliferation and migration when it was added in the presence of endometrial PF. These studies suggest that CXCR4 may be working upstream of EZH2 and may be regulating its expression. However, other studies have shown that the expression of H3K27me3 has a direct effect on the expression of CXCR4 and when the expression of H3K27me3 was lost, this effect was lost as well and CXCR4 expression was increased [237]. Thus, further studies are needed to understand the PF-mediated epigenetic regulation of the CXCR4 axis.

The studies in this thesis, along with other prior studies from our laboratory, point to the PF from women with endometriosis and the inflammatory molecules that are present within that PF, influencing the mechanisms at play in endometriosis. Our results presented here also suggest that targeting one specific mechanism in endometriosis may not be the best option, but rather a combination targeting due to the complex nature of endometriosis. Future studies will examine the effects of these compounds in an animal model to test for decrease in lesion growth, as well as pain.

While endometriosis is not considered a cancer, there are many similarities between the two diseases. The most glaring of them is growth of endometriotic lesions in locations outside their normal location (endometrium/uterus). Endometriotic lesions can also undergo angiogenesis (develop their own blood supply). Cell adhesion factors and immunobiological factors have also been seen to be altered in both endometriosis and cancer [63]. In cancer, many physicians are turning towards precision and personalized medicine (PPM) for treatment of their patients. The idea behind PPM is to provide treatments for a single patient or a single type of cancer rather than treating cancer as a whole as it has been seen that all cancer is not created equally. To develop this type of medicine and treatment option, researchers have taken biopsies from certain cancers and compared the genes in those cancer cells to normal cells and noted specific differences. To be targeted to the cancer, therapies and treatments are then developed and given to either shrink or eliminate that particular cancer. This can also be done on a person-by-person basis where a gene that is not normally targeted in certain cancer treatments may now be added into that patient's treatment plan [246]. It is our hope that by increasing the knowledge of the mechanisms involved and targets that are at play in endometriosis, this same concept of PPM can be adapted for this disease. This would provide not only a treatment tailored towards the

symptoms that women with endometriosis experience, but also reduce the lesions that are present without having to resort to surgery.

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APPENDIX A: IRB LETTER

April 14, 2021

Nalini Santanam, Ph.D, MPH
Department of Pharmacology, Marshall University

RE: IRBNet ID# 114954-26
At: Marshall University Institutional Review Board #1 (Medical)

Dear Dr. Santanam:

Protocol Title:	[114954-26] (9074) Pain Molecules in the Peritoneal Fluid of Women with Endometriosis
Expiration Date:	May 14, 2022
Site Location:	MU
Submission Type:	Continuing Review/Progress Report APPROVED
Review Type:	Full Committee Review

The above study was approved for an additional 12 months at today's full board convened meeting of the Marshall University Institutional Review Board #1 (Medical). The approval will expire May 14, 2022. Since this approval is within 30 days of the expiration date, the fixed anniversary date of 5/14 was maintained. Continuing review materials should be submitted no later than the deadline for the 5/11/2022 IRB agenda.

Please note you are nearing the stated maximum enrollment goal of 300. It was suggested the study be amended if you wish to continue past that maximum.

If you have any questions, please contact the Marshall University Institutional Review Board #1 (Medical) Coordinator Margaret Hardy at (304) 696-6322 or hardyma@marshall.edu. Please include your study title and reference number in all correspondence with this office.

Sincerely,

Bruce F. Day, ThD, CIP
Director, Office of Research Integrity

APPENDIX B: ABBREVIATIONS

μg: microgram

μL: microliter

μM: micromole

ADAM19: A Disintegrin and Metalloproteinase 19

AEBP2: AE Binding Protein 2

ANOVA: Analysis of variance

ARID1A: AT-Rich Interaction Domain 1A

AZA: 5-azacytidine

BAF250a: BRG-associated factor 250a

BANCR: BRAF-associated non-coding RNA

BDNF: Brain-derived neurotrophic factor

BRCA1: breast cancer type 1 susceptibility protein

CA125: cancer antigen 125

CARM1: Coactivator Associated Arginine Methyltransferase

CBX: Chromobox

CCL25: C-C Motif Chemokine Ligand 25

CD8A: Cluster of Differentiation 8a

cDNA: complementary Deoxyribonucleic acid

CEBPB: CCAAT Enhancer Binding Protein Beta

ChIP: Chromatin Immunoprecipitation

CI: cell impedance

Cox-2: cyclooxygenase-2

CS-FBS: charcoal-stripped fetal bovine serum

CXCL12: C-X-C Motif Chemokine Ligand 12

CXCR4: C-X-C Motif Chemokine Receptor 4

CXCR7: C-X-C Motif Chemokine Receptor 7

DNA: Deoxyribonucleic acid

DNMT: DNA methyltransferase

DIE: deep infiltrating endometriosis

DLBCL: diffuse B-cell lymphoma

Dot1L: DOT1-like protein

DPP4: Dipeptidyl peptidase 4

DRG: dorsal root ganglion

DZNep: 3-Deazaneplanocin A hydrochloride

EAMs: endometriosis associated macrophages

EC: endometrial cells

EcE: ectopic endometriotic tissue

EEC: endometrial epithelial cell

EED: Embryonic Ectoderm Development

EMT: epithelial-to-mesenchymal transition

EP3: Prostaglandin E receptor 3

EP4: Prostaglandin E receptor 4

ERβ: estrogen receptor beta

ESC: embryonic stem cells

EuE: eutopic tissue from endometriosis patients

EuN: eutopic tissue from control patients

EZH1: Enhancer of Zeste Homolog 1

EZH2: Enhancer of Zeste Homolog 2

FOXP3: forkhead box P3

GnRH: Gonadotropin-releasing hormone

H3: Histone 3

H3K9: Histone 3 lysine 9

H3K9me2: Histone 3 lysine 9 di-methylation

H3K27: Histone 3 lysine 27

H3K27me3: Histone 3 lysine 27 tri-methylation

H3K79: Histone 3 lysine 79

H4: Histone 4

H4K16: Histone 4 lysine 16

H19: H19 imprinted maternally expressed transcript

HATs: histone acetyltransferases

HBSS: Hanks' Balanced Salt solution

HDACs: histone deacetylases

HDL: high-density lipoprotein

HMTs: histone methyltransferases

HOXA10: homeobox A10

HP-1: heterochromatin-like protein 1

HRP: Horseradish peroxidase

ICAM: intercellular adhesion molecule

IGF-1: Insulin Like Growth Factor 1

IL4R: interleukin-4 receptor

IL-1: interleukin 1

IL-6: interleukin 6

IL-8: interleukin 8

IL-18: interleukin 18

IL-21: interleukin 21

IL-22: interleukin 22

IP: immunoprecipitation

JARDI2: Jumonji and AT-Rich Interaction Domain Containing 2

JmjC: Jumonji C

JUN: Jun Proto-Oncogene, AP-1 Transcription Factor Subunit

kDa: kilodalton

KLF-12: Kruppel like factor 12

LDL: low-density lipoprotein

Let-7: lethal-7

lncRNA: long non-coding RNA

LpMs: large peritoneal macrophages

LSD1: lysine demethylase 1

MAPK14: Mitogen-activated protein kinase 12

MCP-1: monocyte chemotactic protein-1

MDBs: methyl-CpG binding proteins

MDMs: monocyte-derived macrophages

MeCP2: Methyl-CpG Binding Protein 2

mg: milligram

MICB: MHC class I polypeptide-related sequence B

miRNA: microRNA

MMP9: Matrix metallopeptidase

mRNA: messenger ribonucleic acid

NAG-1: nonsteroidal anti-inflammatory drug activated gene 1

NSAIDs: nonsteroidal anti-inflammatory drugs

p21: cyclin-dependent kinase inhibitor 1

p53: tumor protein P53

PBS: Phosphate Buffer Saline

PC: peritoneal cavity

PcG: polycomb

PCR: Polymerase Chain Reaction

PDCD4: Programmed Cell Death 4

PF: peritoneal fluid

PGE2: Prostaglandin E_2

PGF2: Prostaglandin F_2

PHF1: PHD finger protein 1

PHF19: PHD finger protein 19

PI3K: phosphatidylinositol-3-kinase

PhoRC: Pho repressive complex

PR-B: progesterone receptor B

PRC1: Polycomb repressive complex 1

PRC2: Polycomb repressive complex 2

PTEN: phosphatase and tensin homolog

qPCR: quantitative Polymerase Chain Reaction

RbAp46/48: Retinoblastoma protein associated protein 46/48

RIPA: Radioimmunoprecipitation assay buffer

RNA: Ribonucleic Acid

RT-qPCR: Real Time- quantitative Polymerase Chain Reaction

SCR: scavenger receptor

SEM: standard error of the mean

SF-1: steroidogenic factor-1

SIRT1: sirtuin 1

SMAD: SMAD Family Member

SUZ12: Suppressor of Zeste 12

SWI/SNF: SWItch/Sucrose Non-Fermentable

TBST: Tris-buffered saline with Tween 20

TGF-β: transforming growth factor beta

Th17: T helper 17

TNF-α: tumor necrosis factor alpha

Tregs: regulatory T-cells

TrxG: trithorax

TSA: Trichostatin A

VEGF: vascular endothelial growth factor

VEGFB: vascular endothelial growth factor B

VEGFR2: vascular endothelial growth factor receptor 2

VPA: valproic acid

WES: automated western blotting system

ZEB2: Zinc Finger E-Box Binding Homeobox 2